# THE INITIATION AND CONTROL OF YEAST SPORULATION

by

GEOFFREY R CALVERT

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D.G.
Geoffrey R Calvert

CONTENTS

# ILLUSTRATIONS

SUMMARY

Several aspects of the control of sporulation in Saccharomyces cerevisiae have been examined.

The close connection between respiratory capability and ability to sporulate was demonstrated by experiments on several phenotype characteristics of the spd1 (sporulation derepressed) mutant (Dawes, 1975), including its hypersporulation on several non-fermentable carbon substrates, its reduced oxygen consumption and its ability to grow on the fermentable substrate dihydroxyacetone, a derivative of glycerol, which is non-fermentable and on which spd1 mutants hypersporulate and fail to grow. These mutants were also unable to reduce the dye TTC (triphenyl tetrazolium chloride) often used as a test for respiratory capability.

Three asporogenous initiation (spo0) mutants were isolated, both from spd1 and wild-type parent strains, and shown not to sporulate under any conditions tested. Those spo0 mutants derived from spd1-carrying strains had regained the ability to grow on non-fermentable carbon substrates and the ability to reduce TTC, which they also reduced under conditions in which the wild-type did not. These mutants showed an aberrant morphology, and marked loss of viability under starvation conditions.

There was some evidence of loose linkage between one spo0 mutation, designated spo53, and spd1, but otherwise the three spo0 mutations were independent of, and unlinked to, the spd1 or spd3 mutations and a number of other mutations, including cdc25, cdc28 and cdc35 (Hartwell, 1974; Shilo et al.,1978) and whi1 and whi2 (Sudbery et al., 1980).

The interaction of the spd1 mutation and the cell-size control mutations whi1 and whi2 (Sudbery et al., 1980) was examined, using strains carrying each mutation individually and also double mutants, which were found to be intermediate in size between spd1 and whi mutants. Experiments on the ability of/

/different sized cells to sporulate indicated that cells smaller than 4.5μm in length did not sporulate. Further experiments using continuous cultivation techniques showed that wild-type cells smaller than 4.5μm in length did not sporulate, and that this reflected a critical cell volume of 26$\mu m^3$.

Stable continuous cultures of sporulating yeast were obtained in which the percentage of asci and the percentage of budded cells were found to vary over a range of dilution rates, and which showed a marked difference between the behaviour of wild type cells and spd1 mutant cells.

Using a rabbit reticulocyte in vitro protein synthesis system primed with RNA from cells at various stages of sporulation, the appearance of several new mRNA species was shown to occur during sporulation, at least one of which only appeared in sporulating cells. This indicates that there is presumably de novo synthesis of proteins during sporulation and, therefore, control at the level of transcription.

ABBREVIATIONS USED

| | |
|---|---|
| $A_{280}$ | Turbidity (Absorbance) at 280nm |
| $A_{600}$ | Turbidity (Absorbance) at 600nm |
| cAMP | Cyclic adenosine monophosphate |
| ATP | Adenosine triphosphate |
| CP | Creatine phosphate |
| CPK | Creatine phosphokinase |
| DHA | Dihydroxyacetone |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| EDTA | Ethylene diaminotetraacetic acid |
| EGTA | Ethylene glycol-bis-( -aminoethyl ether)N, N' - tetraacetic acid |
| GDH | Glutamate dehydrogenase |
| HEPES | 4(2 - hydroxyethyl)-1-piperazineethane sulphonic acid |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Reduced nicotinamide adenine dinucleotide |
| POPOP | 1,4-bis[5-phenyl-2-oxazolyl] benzene |
| PPO | 2,5 diphenyloxazole |
| RNA | Ribonucleic acid |
| mRNA | Messenger ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| tRNA | Transfer ribonucleic acid |
| spd | Sporulation derepressed (mutation/mutant) |
| spo | Asporogenous (mutation/mutant) |
| TCA | Trichloroacetic acid |
| TCAcycle | Tricarboxylic (Krebs)cycle |
| TEMED | N,N, N' , N' , Tetramethylenediamine |
| TTC | Triphenyl tetrazolium chloride |

CHAPTER 1

INTRODUCTION

The task of the microbiologist is to study how cells function in their environment. The information gained through biochemical, cytological and genetical studies is fundamental to the understanding of cell function, but to study the cell as a whole, one needs to know how the individidual processes interact and enable the cell to control and change its form and function in response to external and internal stimuli. With recent developments in the understanding of genetical and biochemical processes, it is important that morphogenetic studies should be undertaken to relate individual processes to the complex interactive aspects of cell behaviour.

Meiosis and sporulation in the yeast Saccharomyces cerevisiae form an excellent model morphogenetic system. Yeast can be manipulated easily by standard microbiological techniques, and its genetics are made easier to study by the presence of stable haploid and diploid phases in the life cycle, meaning that mating, complementation, dominance and linkage tests can be performed by meiotic segregation. Yeast is well understood biochemically and genetically. (For general reviews of most aspects, see "The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance" Strathern, Jones & Broach (eds), Cold Spring Harbor Laboratory, 1981). The sporulation process also commends itself as a morphogenetic model as it is simple, reproducible and can be initiated synchronously by few, defined, events. General reviews of sporulation in yeast include: Fowell (1975); Tingle et al., (1973) for physiological aspects, and Esposito & Esposito (1974, 1975) and Esposito & Klapholz (1981) for genetic aspects.

The general aim of this thesis is to examine the regulation of sporulation, particularly in regard to the initiation events, and the introductory pages that follow review the current state of knowledge in the field. First, a/

/brief account of the life cycle and homothallism is given to place the sporulation process in the overall functioning of the cell, and secondly the events which occur during the process are discussed.

Lastly, and most importantly for our purposes here, the regulation of sporulation is discussed.

## THE LIFE CYCLE OF YEAST

The life cycle of Saccharomyces cerevisiae is shown in figure 1.1. The mitotic growth cycle, shown at top and bottom, occurs in both haploid and diploid forms during which reproduction is by budding. Haploid cells can be of a or α mating type, carrying the MATa or MAT α gene respectively at the mating type locus, and diploids are formed by the mating of a and α to form a stable zygote which buds out to form a stable diploid vegetative cell.

Haploids form from diploids via the sporulation process. Under defined starvation conditions (see p.25 ) diploid cells cease to initiate growth by budding and mitosis, but proceed to meiosis, and the four meiotic genomes so produced are packaged into individual dormant spores. For a general review of the life cycle, see Mortimer & Hawthorne (1969) and for sporulation see Fowell (1975). For a review of the cytology of the mitotic cycle, see Byers (1981) and for the molecular biology of the cell cycle, see Pringle & Hartwell (1981).

### Mating and homothallism

Cells of the α mating type can produce an α mating pheromone which arrests a cells in the cell cycle prior to the initiation of DNA synthesis, and induces them to elongate (Bucking-Throm et al., 1973; Mackay & Manney, 1974). Cells of the mating type produce a similar pheromone. (Crandall et al., 1977). The particular mating type genes present control a number of cell functions, expression of some genes and sporulation (see Herskowitz & Oshima, 1981 for a general review, also see p.41 this thesis).

Mating and the haplophase in Saccharomyces cerevisiae were discovered when it was widely thought that this organism was exclusively homothallic that is, able to undergo sexual/

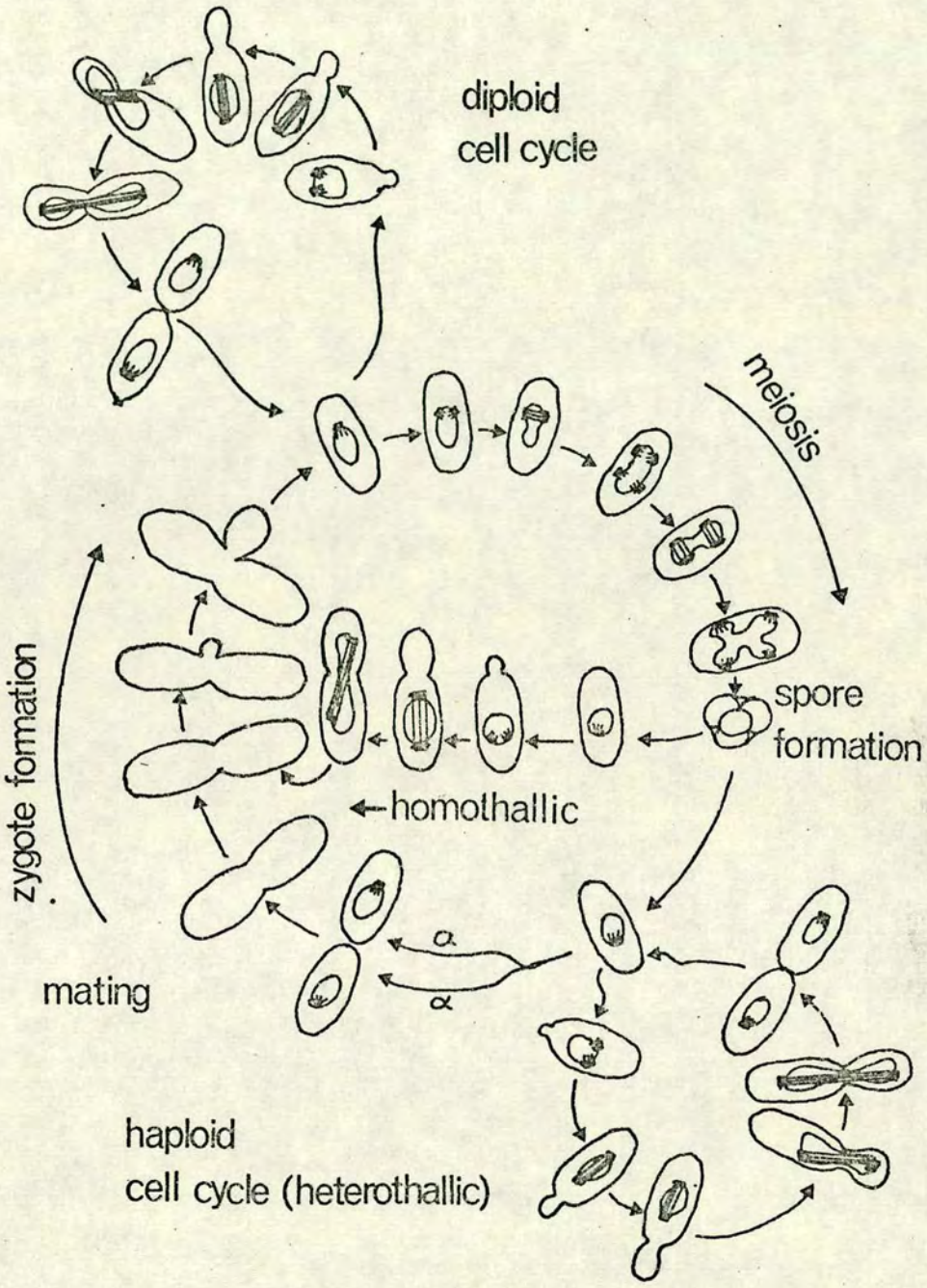


Figure 1.1 Life cycle of Saccharomyces cerevisiae.
Configuration of spindle apparatus shown.

/reproduction without prior mating with another strain (see Fowell, 1969). Homothallic strains are widely used, for example, in studying sporulation; they do not have a stable mitotic haplophase. Spores of homothallic strains, after germination and the first division can change their mating type and mate with the sister nucleus to form a diploid (Hicks & Herskowitz, 1976).

To exaplain the ability of homothallic strains to change their mating-type, a "cassette" model has been proposed (Harashima & Oshima, 1976; Hicks et al., 1977; Hicks & Herskowitz, 1979). Genetic studies revealed in $\alpha$ strains the HMR$a$ locus which contains silent $a$ -specific information required for the transition to $a$ mating-type. The HML $\alpha$ locus provides $\alpha$ information to switch $a$ cells to $\alpha$ . It has been shown that during the mating type "switch", copies of the "silent" information are inserted by a specific recombination process into the mating type locus. The ability to perform this "switch" is conferred by the HO gene which is unlinked to the mating type locus. The major characteristics of the "cassette" model are shown in figure 1.2 (After Herskowitz & Oshima, 1981).

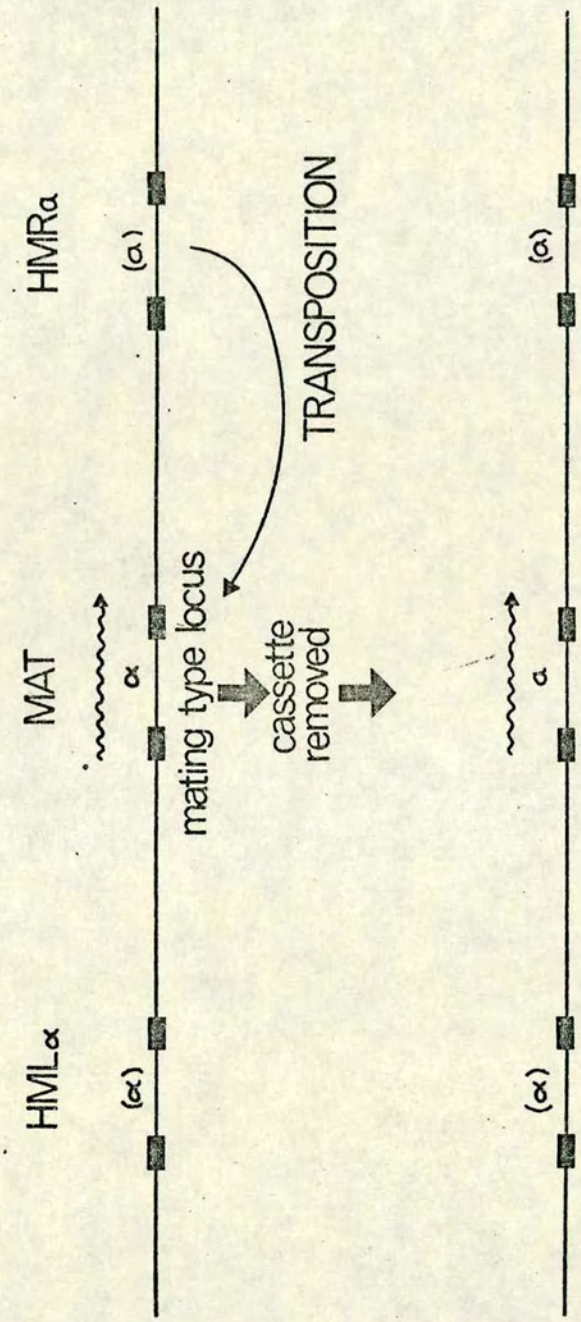


Figure 1.2 The "cassette" model of mating type interchange in homothallism.
Redrawn from Herskowitz & Oshima (1981)

## MEIOSIS AND SPORULATION

Vegetative growth of yeast is by mitosis and budding. The nucleus and division apparatus are substantially similar to those of higher eukaryotes, with a nuclear envelope, nucleolus, most of the histones and the spindle apparatus (Matile et al., 1969). A major difference is that the nuclear envelope does not break down during mitosis, but envelopes the daughter nucleus as it is formed (Robinow & Marak, 1966).

As shown previously, cells in the diplophase can, under certain conditions, initiate meiosis and sporulation. Figure 1.3 shows the general requirements for the initiation of meiosis and sporulation. These are, briefly, that the cell must be in that part of the cell cycle in G1 between mitosis and the "start" event (Hartwell, 1974), that the cell must have derepressed mitochondrial activities (see p.23 ), that both alleles of the mating type locus must normally be present (Roman & Sands, 1953; p.41 this thesis) and that high levels of readily metabolized sugars and/or readily available nitrogen sources must not be present (see p.28 ). Mutants are known in which all these requirements are modified, so they cannot be regarded as absolute.

### The cytology of sporulation

Detailed information on cytological stages of the sporulation process has been obtained, particularly from the serial thin-section work of Moens (1971) and Moens & Rapport (1971 a, b), the freeze-etch electron micrographs of Hashimoto et al. (1960) and more recent work on chromosome behaviour by Peterson & Ris (1976). Figure 1.4 shows the process of sporulation following the nine-stage scheme of Esposito & Esposito (1975) with an additional tenth stage to include deposition of the outer spore coat, following Dawes (1981).

Table 1.1 Requirements for the Initiation of Meiosis and Spore Formation (after Dawes, 1983)

(i) Heterozygosity at Mating-Type Locus

MATa/MATα, MATa/MATa/MATα, MATa/MATa/MATα/MATα all sporulate.

MATa, MATα, MATa/MATa, MATα/MATα, MATa/MATa/MATa etc. do not.

(ii) Completion of Mitotic Cell Division

Sporulation is initiated only in the G1 phase of the cell cycle.

(iii) Nutrient Conditions

(a) Depletion of limitation of the carbon source, mitochondrial induction probably necessary.

(b) Depletion or limitation of the nitrogen source, especially $NH_4^+$.

Figure 1.4 The cytology of meiosis and sporulation in yeast. Redrawn after Dawes (1981)

Structures represented are: cell wall (cw); endoplasmic reticulum (er); nucleus (n); nucleolus (no); mitochondrion (m); spindle plaque (sp); spindle microtubule (sm); lipid granule (lg); vacuole (v); synaptonemal complexes (sx); polycomplex body (pb); spore coat (sc); outer spore coat (osc).

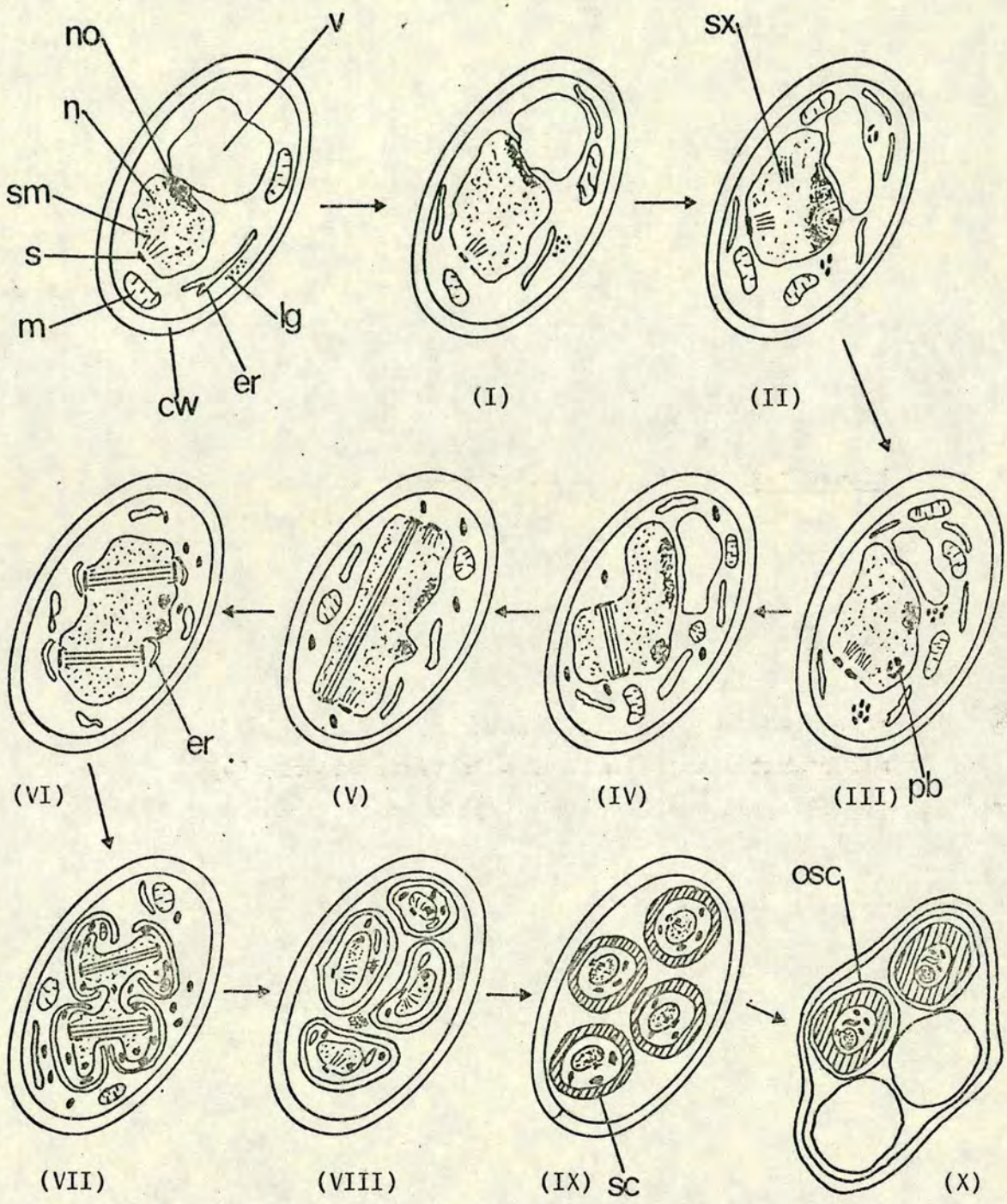


Figure 1.4 the cytology of sporulation.
See opposite for key to symbols.

At Stage I, the cell is starved and vacuolate, with a single spindle pole body attached to the nuclear membrane. During Stage II a "polycomplex" body containing multiple synaptonemal complex-like elements appears in the nucleus, and during the following Stage III the spindle pole body divides. Stage IV includes the commencement of the first meiotic division, with the two spindle pole bodies becoming opposed on the nuclear membrane and connected by a spindle apparatus of microtubules. At this stage the synaptonemal complex elements of the polycomplex body disappear. During Stages I - IV the vacuole decreases in size and may fragment, and lipid granules begin to increase in size and number. In Stage V meiosis I ends, with elongation of the nucleus and duplication of the two opposed spindle pole bodies. These newly-formed spindle pole bodies migrate to oppose each other during stage VI and two meiosis II sets of spindle apparatus are formed. During this stage the endoplasmic reticulum forms a double membrane layer associated at first with the spindle pole bodies, which eventually forms the prospore wall in later Stages. The spindles extend during Stage VII and the nucleus becomes highly lobed, although through all these stages the nuclear membrane remains single, continuous and intact. The prospore wall membranes begin to surround the lobes of the nucleus. In Stage VIII the enclosure of the lobes is complete and the spore nuclei become separate. Here the spindle pole body also detaches from the prospore wall. In Stage IX the synthesis of spore coat material occurs between the two membranes derived from the endoplasmic reticulum. In the last Stage X as added by Dawes (1981), an outer spore coat is formed which is thin and electron-dense, appearing from the electrophoretic evidence of Briley et al. (1970) to be largely composed of protein, and probably containing the determinant of the spore surface antigen (Snider & Miller, 1966).

## Biochemical events during sporulation

There are surprisingly few biochemical events known to/

/occur only in sporulating cells. By describing an event as sporulation-specific, it is generally understood that it does not occur in non-sporulating but otherwise isogenic strains, normally a/a or α/α diploids. It seems that the cell, when preparing for dormancy in the sporulated condition, uses fully the existing mechanisms for producing dormancy under starvation, and therefore many events are common to the two processes. Those events that are considered to be specific to sporulation include:

(i) DNA synthesis The first biochemical indication that sporulation has been initiated is premeiotic DNA synthesis, doubling the chromosome complement from 2n to 4n. This begins after about four hours and is complete by ten hours at the latest, in preparation for the first meiotic division (Croes, 1966; Esposito et al., 1969; Roth & Lusnak, 1970; Sando & Miyake, 1971; Simchen et al., 1972). The DNA synthesized does not appear to be functionally distinct from mitotic 4n DNA since the cell can proceed to a normal mitotic division if returned to vegetative growth medium (Simchen et al., 1972). Many functions of mitotic DNA synthesis are common with those of premeiotic DNA synthesis as some cdc mutations affect both (Simchen, 1974).

There is some evidence for the existence of functions used in premeiotic DNA synthesis that are not used in mitotic DNA synthesis. First, the distinctive pattern and rate of premeiotic DNA synthesis (Williamson et al., 1980) indicates unique functions, although it is far from clear whether this effect is due to starvation or is sporulation specific. Secondly, several mutations have been isolated which cause defects in premeiotic, but not mitotic DNA synthesis (Esposito & Esposito, 1973; 1974b). Thirdly, a/a and α/α cells fail to undergo premeiotic DNA synthesis, although they are perfectly well able to undergo mitotic DNA synthesis (Roth & Lusnak, 1970; Roman & Sands, 1953; Haber et al., 1975).

(ii) Genetic recombination During premeiotic DNA synthesis, wild type cells acquire an enhanced ability to undergo intragenic (Sherman & Roman, 1963) and intergenic (Esposito & Esposito, 1974) recombination, compared with the low level in mitotically dividing cells (Hartwell, 1974). This increase in recombination ability can be detected close to the start of premeiotic DNA synthesis, and is evidently under some form of co-ordinate control (Roth, 1973). Enhanced recombination is not, however, an integral or essential result of premeiotic DNA synthesis, as cells carrying the CSP1 mutation, which allows low levels of sporulation of a/a and α/α diploids, can exhibit premeiotic DNA synthesis without recombination. Those cells which do sporulate, however, do display enhanced recombination (Hall & Hopper, 1973; Hopper et al., 1975). Also rad 50-1 spo 13-1 double mutants can form viable ascospores without recombination, though with an incomplete meiosis (Klapholz & Esposito, 1980).

Mutants defective at three loci, con 1, con 2, and con 3 are defective in intragenic recombination but sporulate normally (Fogel & Roth, 1974).

Cells which are returned to rich medium before they are committed to meiotic segregation still show enhanced recombination levels (Esposito & Esposito, 1974b; Esposito et al., 1974), however these cells have undergone premeiotic DNA synthesis.

It thus appears that enhanced recombination is normally associated directly with premeiotic DNA synthesis, but though the latter can occur without enhanced recombination, enhanced recombination cannot be shown to occur in the absence of premeiotic DNA synthesis.

(iii) RNA synthesis Bulk RNA content of sporulating cells increases between four and six hours after resuspension in sporulation medium (Sando & Miyake, 1971; Esposito et al., 1969), after which there is a steady decrease until the formation of/

/mature ascospores indicating limited RNA synthesis during this period. The lack of suitable inhibitors has prevented a clear indication of whether RNA synthesis is essential for sporulation. The pattern of RNA synthesis, in sporulating and isogenic non-sporulating cells, is identical in terms of the different classes of RNA (Tingle et al., 1973) except for a 20S RNA which accumulates only in sporulating cells (Sogin et al., 1975). This 20S RNA accumulates during sporulation, contained in a 32S ribonucleo-protein particle, the protein component of which has also been shown to be specifically synthesised during sporulation, although the particle does not appear in all sporulating strains, so it's significance is unclear (Wejksnora & Haber, 1978).

The general rate of rRNA synthesis is much slower during sporulation (Sogin et al., 1975). The incorporation of labelled phosphate into the 27S and 20S precursors was approximately six times slower in sporulating than in vegetative cells, and the rate at which these precursors were processed into mature ribosomal RNA was ten times slower in sporulating cells. Temperature-sensitive rRNA synthesis mutants were found by Haber (1975) to be temperature sensitive for sporulation, indicating a requirement for rRNA synthesis during sporulation. However, rRNA continued to accumulate in these strains, complicating the picture.

Transfer RNA is synthesized during sporulation and Sogin et al. (1975) have shown that this is not due to accumulation of a single, or a small number, of species. The question of whether there are sporulation-specific differences in the accumulation of specific species of tRNA has not been fully answered.

The question of mRNA synthesis during sporulation is important as it bears upon the main subject of this thesis, the regulation of sporulation. If there is regulation at the genetic level, new gene expression is required. The work of/

/Esposito & Esposito (1969, 1975) on asporogenous mutants would indicate that new gene expression occurs, but in fact does not prove it as the genes may be expressed continuously but their products are only essential at a particular stage of sporulation. Evidence is accumulating (see following section) that there are new sporulation-specific proteins produced, but it is not known whether these are synthesized de novo or by modification of pre-existing proteins. A later chapter in this thesis will demonstrate that sporulation-specific new gene expression does occur and that at least one, and probably several, new messenger RNAs are produced specifically during sporulation.

(iv) Protein synthesis Bulk protein synthesis is continuous throughout sporulation, as shown by incorporation of ( $^{14}C$) amino acids into hot TCA-precipitable material (Esposito et al., 1969). There were two peak rates of synthesis, one at 4-6h into sporulation, and the second at 16-20h, at the stage of ascospore maturation. Total protein content of the cells increases during Stages I - III and decreases, showing that there is continued protein degradation as well as synthesis (Esposito et al., 1969). Protein synthesis is essential at all stages since addition of cycloheximide at any stage prior to the appearance of mature ascospores halts the process (Esposito et al., 1969).

Although protein synthesis is thus seen to be essential, the situation is somewhat different with regard to the specificity of protein synthesis. Snider & Miller (1966) showed the presence of a novel antigenic component in the spore coat unique to sporulating cells, and there is some evidence that this is at least partly due to a protein component (Dawes et al., 1983).

Magee (1974) has preliminary data indicating the appearance of a new RNA polymerase activity specific to sporulation, however these are concerned solely with elution patterns of RNA polymerases from ion-exchange columns and more detailed confirmation of the change has not appeared.

Matur & Berry (1978) studied five enzymes whose activities increased as a 'step' during sporulation. All but one of these, however, also showed a 'step' increase in activity during vegetative growth, the exceptions being proteinase A. del-Rey et al. (1979) showed the appearance of a new 1-3β glucanase during sporulation, involved with the mobilization of carbohydrate reserves, and this appearance was sporulation-specific. Again however, this work shares the problem of only being able to show a change in activity, and not a change in protein level or mRNA level.

Colunna & Magee (1978) and Clancy et al.(1980) have found a new α-glucosidase activity which appears late in sporulation and only in a/α cells. This new activity is due to new protein synthesis, as shown by antibody studies (Magee, pers. comm.)

Initial attemps to show the synthesis of new polypeptide species during sporulation, by pulse-labelling sporulating cells with labelled amino acids, followed by one-dimensional (Hopper et al., 1974) and two-dimensional (Trew et al., 1979) gel electrophoresis, were unable to detect the synthesis of any new sporulation-specific proteins. Dawes et al. (1980) however, were able to show that sporulating cells became progressively less able to take up amino acids, and that most of the label in a pulse was taken up by a small proportion of non-sporulating cells. Wright & Dawes (1979), by a pre-labelling procedure, fully and homogeneously labelled cell proteins, and then initiated the cells to sporulation. This procedure showed a number of new sporulation-specific polypeptides appearing during sporulation, as detected by two-dimensional polyacrylamide gel electrophoresis followed by autoradiography. Ajam et al. (1981) and Wright et al. (1981) showed that these changes occurred in an orderly, timed sequence, indicating a close degree of control. However, this system cannot readily elucidate the contribution of either de novo synthesis or modification of pre-existing proteins. Ajam (1981) classified some changes on the basis of differences/

/observed in cycloheximide-treated cultures, and showed that some early changes depend on de novo synthesis on cytoplasmic ribosomes, whereas some other sporulation-specific changes occurred in the presence of cycloheximide and may therefore have been due to modification.

(v) Degradation of RNA and Protein Although protein synthesis is essential for sporulation, the process can occur in the absence of exogenous nitrogen compounds and, in the case of auxotrophic mutants, in the absence of specific amino acid requirements (Esposito et al., 1969; Halvorson, 1958 a,b), indicating that protein synthesis can be supported by the intracellular pools, and the products of protein degradation. Klar & Halvorson (1975) showed that protein degradation is extensive, with a minimum of 30% of cell protein degraded during sporulation. Three proteinases, A, B and C, were found to increase in a sporulation specific, and cycloheximide dependent manner, but the actual rate of degradation of protein was not severely affected by cycloheximide (Klar & Halvorson, 1975; Magee & Hopper, 1974). Several protease-negative strains are unable to sporulate (Chen & Miller, 1968; Betz, 1979; Zubenko & Jones, 1981), indicating the fundamental importance of several protease activities in providing amino acids for further protein synthesis. The intracellular amino acid pool is reduced very soon after the onset of sporulation (Ramirez & Miller, 1964) and soon becomes the factor limiting the rate of protein synthesis, so any failure to make available more amino acids will result in blockage of protein synthesis and therefore sporulation. Increased protein turnover is also a feature of starved vegetative cells, indicating again a similarity between vegetative stationary phase and sporulation.

Extensive RNA breakdown occurs during sporulation, beginning immediately after initiation, and continuing at a high rate until ascospore maturation. This increase in RNA degradation does not occur in similarly treated a/a or α/α strains (Hopper et al., 1974).

After about 24h in sporulation medium, a/α cells degraded 50-70% of cellular RNA to free acid soluble material, with a/a and α/α strains only degraded about 20%. This does not take into account turnover of RNA and reuse of precursors for further RNA synthesis, implying that the real breakdown rate was much greater.

The functional significance of increased protein and RNA degradation has not been fully established, although there are several plausible explanations:

(i) Turnover is necessary to provide new monomers for sporulation-specific macromolecules. Although this is doubtless the case, it is of limited value in explaining the extent of turnover, since a large part of the degraded material is excreted (Hopper et al., 1974).

(ii) Unwanted biologically active macromolecules are removed from the cell in preparation for dormancy. This view is supported by the apparently wasteful excretion of monomers.

(iii) When the developing spores are in an advanced stage of differentiation, macromolecules may be unable to cross the enveloping membranes and the parent cell macromolecules must be broken down to provide monomers for the spores' own biosynthetic apparatus. This would ensure that, at least in certain respects, the spore's biochemical make-up would be a function of its own, and not the mother cell's genotype. Magee (1974) has shown that in cells heterozygous for a cyh$^r$ (recessive cycloheximide resistance) mutation, partial cycloheximide resistance appears before full spore maturation, indicating that at an earlier stage the spore ribosomes have been made from the spores' own genetic information.

If is not clear to what extent the high rates of protein and RNA breakdown, though specific to sporulation, are essential to it.

(vi) Glycogen metabolism Formation of carbohydrate is a major activity in sporulating cells, accouting for around two thirds of the total increase in cell mass (Kane & Roth, 1974). However, the changes in soluble glycogen content are the only ones specific to sporulating cells. Pontefract & Miller (1962) were able, by cytochemical methods, to show glycogen synthesis early in differentiation, followed by degradation during maturation. Kane & Roth (1974) found that glycogen synthesis is not specific to sporulation whereas glycogen breakdown is. Glycogen breakdown also appears to be specifically associated with spore maturation as in strains sporulating at different times, glycogen is always degraded at this stage. Both glycogen synthesis and breakdown are dependent on protein synthesis, and both cease within 1-2h after the addition of cycloheximide (Hopper & Magee, 1974), indicating that there is continuous turnover of glycogen synthetic and degradative enzymes. Colonna & Magee (1978) examined the glycogenolytic enzymes produced during sporulation, and found 1-4 glucosidase and 1-6 glucosidase activities. Glycogenolytic activity was found to be minimal in vegetative cells, and the 1-4 glucosidase activity was shown to be sporulation-specific (see p.19 ).

The significance of glycogen catabolism in sporulating yeast is not clear. It is unlikely to be simply providing glucose for energy metabolism, as it occurs at a time when the respiration rate is low and declining (Hopper et al., 1974), although none of the breakdown products are probably used for this. More likely is that some of the monomers are incorporated into ascus and spore wall structural polysaccharide and some are converted to trehalose for energy storage in the spore.

(vii) Ether resistance The latest known event in sporulation is the development of resistance to diethyl ether. For a mixture of mature ascospores and vegetative cells, a concentration of ether can be found at which nearly all/

/vegetative cells are killed, but at which 80-90% of ascospores survive (Dawes & Hardie, 1974). Ether resistance develops during the maturation process, probably associated with the deposition of the outer spore coat.

Energy Metabolism During Sporulation

Sporulation in Saccharomyces cerevisiae is an energy-intensive process requiring a constant supply of ATP, which is obtained almost exclusively by the catabolism of exogenous energy sources. Cells suspended in distilled water or buffer without an exogenous energy source sporulate very poorly, if at all (Pontefract & Miller, 1962). Early studies suggested a total dependence of sporulation on respiratory capability in the cells; Ephrussi & Hottinguer (1951) showed that $\rho^-$ petite strains, lacking mitochondria and therefore respiratory deficient, were unable to sporulate under any conditions tested. An oxygen supply is essential; sporulation does not occur under anaerobic conditions (Hansen, 1902 cited by Fowell, 1969). Exogenous energy sources are respired (Croes, 1967a; Vezinhet et al., 1969) and an active TCA cycle appears to be normally necessary (Fast, 1973; Roth & Halvorson, 1969; Esposito et al., 1969). The ability of cells to initiate sporulation immediately on inoculation into sporulation medium depends critically on the energy source on which they have been pre-grown. Glucose grown cells have repressed TCA-cycle enzymes (Polakis & Bartley, 1965) and show a considerable lag before sporulating, whereas glycerol or acetate grown cells, which have fully functional TCA cycle activities, show rapid and synchronous sporulation (Fast, 1973; Roth & Halvorson, 1969). Galactose, although a fermentable carbon source, does not repress the TCA cycle, and cells pre-grown on this also show synchronous sporulation. There is some evidence for the involvement of the glyoxylate cycle in sporulation (For a discussion see Tingle et al., 1973) and presumably this cycle is used by the cells for conversion of materials such as acetate into cell material, particularly/

/in gluconeogenesis. Esposito et al. (1969)estimated that cells sporulating on acetate convert about 16% of the acetate into cell components, respire 62% and the remaining 22% stays in the soluble pool.

Recent work, however, has complicated the situation. Some MIT$^-$ mutants which, unlike $\rho^-$ mutants, are respiratory deficient due to a mutation at a single locus in the mitochondrial genome, are able to sporulate (Pratje et al., 1979). Cells mutated anywhere within a short central region of the oxi3 mitochondrial locus (i.e. they lack functional cytochrome oxidase and are therefore respiratory deficient) have less than 1% of the normal respiratory capability, but are still able to sporulate to 15% of the population. The strains, however, take 6-7 days to sporulate, and produce mainly one and two-spored asci, suggesting that they lack sufficient energy to sporulate normally.

# THE CONTROL OF SPORULATION

## Introduction

In the previous sections the morphological and biochemical changes accompanying sporulation have been examined. This section is concerned with the regulation of sporulation. The process is important in the life cycle of yeast, providing both a means of survival under adverse conditions, and a means of sexual reproduction, with the accompanying meiotic recombination leading to the transfer of genes between different cell lines. One would therefore expect that the process would be under tight control by a number of external and internal factors to ensure that it occurs when advantageous to survival and not otherwise. Table(1.1) shows the major factors which influence sporulation in Saccharomyces cerevisiae.

## Control by carbon substrates

Saccharomyces cerevisiae, unlike Bacillus subtilis, is initiatedinto sporulation only when both carbon and nitrogen substrates are depleted (Fowell, 1969). This indicates that sporulation in yeast is not simply initiated in response to the cell's inability to grow, but is regulated by absolute external concentrations of particular nutrients detected by the cell. Fermentable hexose sugars such as glucose, fructose and mannose, and disaccharides such as sucrose and maltose have a severe effect upon sporulation. At concentrations above 0.5% (w/v) they abolish sporulation in wild-type cells, even if the cells are unable to grow, and concentrations as low as 0.05% (w/v) have a measurable effect (Fowell, 1967 & 1969; Miller, 1963b). Galactose, although fermentable, has a lesser effect. Non fermentable carbon substrates such as glycerol, acetate or pyruvate, do not inhibit sporulation. To examine the molecular mechanisms involved, the effect of carbon substrates can be split into the effect on initiation of sporulation, and the effect on completion of the process.

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i) Initiation Fermentable carbon substrates in growth media inhibit the subsequent ability of cells to initiate sporulation. Sporulation of glucose-grown cells harvested in the exponential phase and transferred to sporulation medium is markedly inhibited compared with that obtained by log-phase cells grown on non-fermentable carbon sources such as acetate (Fast, 1973). This effect is closely related to the cell's capacity for oxidative respiration. As discussed previously, some mitochondrial functions are essential for sporulation. The effect of carbon substrates on the cell's ability to initiate sporulation closely relates to the induction of the mitochondrially located TCA cycle enzymes. Glucose and other hexoses which repress sporulation repress TCA cycle enzymes, while acetate and other non-fermentable carbon substrates induce the TCA cycle and also fail to repress sporulation. Galactose, which although fermentable, does not repress TCA cycle enzymes, does not repress sporulation. Kuenzi et al. (1975) have convincingly demonstrated that, although a fully functional mitochondrial genome is not essential for sporulation, the induction of several TCA cycle enzymes, particularly cytochrome oxidase, succinate dehydrogenase, and the glyoxylate cycle enzyme isocitrate lyase, is essential. This effect is probably due to the high demand for respiration in the early stages of sporulation. Respiratory inhibitors such as cyanide or dinitrophenol block sporulation, and a good oxygen supply appears to be essential (Miller & Halpern, 1956). Cells grown on glucose will eventually sporulate if resuspended in sporulation medium, but only after they have undergone adaptation to respiratory metabolism (Kuenzi et al., 1975).

It is not clear at present whether the specific effect of glucose on initiation is directly concerned with regulation of gene expression by a mechanism analogous to that mediated by carbon catabolite repression in E. coli. Tsuboi et al. (1972) reported that high cAMP levels in the external medium partially reversed glucose repression of sporulation, and they suggested the involvement of a cAMP mediated control system. Hartig & Breitenbach (1977) were, however, unable to confirm this, although they did show that cAMP levels fluctuated markedly during sporulation, an effect apparently related to the synthesis of/

/macromolecules.

However, the whole question of cAMP effects remains open to considerable doubt, particularly as the changes in its concentration occur well after initiation.

A more plausible explanation is that early sporulation events require high levels of ATP which can only be maintained by respiration. This is supported by the observations of increased oxygen consumption early in sporulation (Croes, 1967), the requirement for a non-fermentable carbon source (Pontefract & Miller, 1962; Miller, 1963b), and the requirement for induction of respiratory functions. Glucose may, therefore, act by making the cells respiratory-incompetent following their resuspension in sporulation medium. Hartig & Greitenbach (1980) have shown that certain MIT$^-$ mutants with less than 1% of normal respiratory capacity can nevertheless sporulate.

Subsequent Regulation

Glucose also affects sporulation after initiation. Cells which are fully respiratory competent fail to sporulate if resuspended in sporulation medium containing high levels of glucose (Miller, 1963b). Before a certain stage has been reached in the sporulation process, adding high levels of glucose returns the cells to vegetative growth (Esposito & Esposito, 1974). The accuracy with which the point of commitment can be established suggests that glucose is not simply repressing sporulation by repressing the synthesis of respiratory enzymes, since these enzymes, and their corresponding mRNA's would be present in the cytoplasm and capable of respiratory activity for some time after glucose had inhibited mRNA synthesis. The mechanism by which glucose inhibits sporulation once it has been initiated is therefore not clear.

Control by Nitrogenous Compounds

In general, those nutrient conditions which favour growth depress sporulation, and this is seen with nitrogenous compounds as well as carbon sources (Miller, 1963b). In the presence of acetate in sporulation medium, ammonium ions, the most readily assimilated nitrogen source have a severe effect on sporulation; concentrations as low as 2mM prevent sporulation completely (Pinon, 1977). A wide range of amino acids have been tested (Miller, 1963b) and they vary in their ability to repress sporulation, although none is as effective as ammonium ions. Yeast extract also represses sporulation to some extent in acetate media (Fowell, 1975).

Ammonium ions exert their repressive effects well after the initiation of sporulation; Miller (1963a) showed that while low concentrations of ammonia prevented any significant numbers of cells in a population from completing sporulation, most still proceeded as far as the first meiotic division. From further evidence, it has been suggested that ammonium ions may be preventing the induction of enzymes required for spore compartmentation and cell wall synthesis (Fowell, 1975).

Nitrogen-repression of enzyme synthesis Work on nitrogen repressible enzyme systems in yeast has shown that a central role is played by the NADP-linked glutamate dehydrogenase (NADP-GDH). This is the enzyme normally responsible for ammonia assimilation (Grenson et al. 1974). The nitrogen repression of allantoinase, arginase, and several amino acid permeases is relieved in gdhA mutants lacking NADP-GDH (Grenson and Hou, 1972; Dubois et al. 1973). Dubois et al. (1974) suggest that the enzyme molecule itself is the effector, through Bossinger et al.(1974), working on allantoinase, suggested that ammonia repression of this enzyme operated through amino acids. NAPD-GDH is degraded by a specific protease as cells enter carbon-source starvation (Mazon, 1978; Mazon & Hemmings, 1979), and this tight control over the presence of the molecule is also evidence for its role as a regulator.

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For a general review of the area, see Cooper (1980).

Function of NADP-GDH in sporulation The function of NADP-GDH in ammonia-repression of sporulation is not clear. Pinon (1977) showed that methylamine, an analogue of ammonia not known to be a substrate for NADP-GDH, is still effective in repressing sporulation, and he suggested that ammonia iteself is directly involved as a controlling element. Other evidence for separate mechanisms in ammonia-repression of enzyme synthesis and sporulation comes from work on spd mutants (Dawes, 1975) in which ammonia repression of sporulation is relieved. This mutation did not affect the regulation of the synthesis of several enzymes subject to different forms of nitrogen repression (Kinnaird, 1979; Vezinhet et al. 1979; See also p.123 )

Newlon (1979) found no release of ammonia repression of sporulation in glutamate dehydrogenaseless mutants, and suggested that the $NADP^+$-linked enzyme was not involved in the process. Recent work by Dickinson & Dawes (1983), however, indicates that these mutants show a significant reduction in ammonia repression of the initiation of sporulation, although not a corresponding increase in completion of ascospore formation.

It seems likely then, that there is not one overall mechanism of ammonia repression of sporulation, but that ammonia represses different individual enzyme systems operating at different times during the process.

## Cell-Cycle Control - Commitment to Sporulation

Up to a certain time after initiation of sporulation, cells remain uncommitted, that is, under certain conditions they can resume vegetative growth. Sherman & Roman (1963) defined the point of commitment to sporulation as that stage reached by the cell after which, if returned to rich growth medium, it has to complete sporulation before resuming vegetative growth. Simchen et al. (1972) have defined several stages in sporulation in terms of the/

/cell's response to the external medium. During the first three hours after initiation, cells will complete sporulation if resuspended in distilled water, indicating that up to that point some events induced by sporulation conditions and necessary to the process have not been completed. After this point, cells will not complete sporulation if resuspended in distilled water, but will still resume vegetative growth if resuspended in rich growth medium, that is, they have completed all the processes on which sporulation is dependent, but have not undergone any committing them to sporulation. After seven hours, cells become incapable of resuming vegetative growth. There is a short transition period of about half an hour, during which cells, if resuspended in rich medium, will neither sporulate nor resume vegetative growth. After seven and a half hours, cells will complete sporulation in rich growth medium. What these results indicate about the time-course of sporulation is that after three hours the last event necessary for sporulation and requiring an external carbon source is completed, after seven hours the first event incompatible with further vegetative growth occurs, and after seven and a half hours the last event necessary for sporulation and repressed by rich growth medium occurs.

The three hour stage coincides with the beginning of premeiotic DNA synthesis, and the seven hour stage with the commencement of meiotic segregation (Esposito & Esposito, 1974). Cells become committed to meiotic recombination during premeiotic DNA synthesis (Esposito & Esposito, 1974), but can still undergo mitotic segregation. Hirschberg & Simchen (1977) suggest that commitment to meiosis occurs at spindle plaque duplication, when the cell has decisively constructed a meiotic, rather than a mitotic apparatus.

Meiotic recombination can be followed by mitotic segregation, however meiotic segregation requires meiotic recombination. (Haber & Halvorson, 1972; Hopper et al. 1975; Hopper & Hall, 1975). Malone and Esposito (1981) have shown, however, that a double mutant containing rad 50-1 (a recombination-deficient mutation (Prakash et al., 1980)), and spo 13-1 (an asporogenous mutant (Klapholz & Esposito, 1980)), can form/

/viable diploid ascospores after a single meiosis II - like equational division without undergoing meiotic recombination, indicating that it is probably only meiosis I that is dependent on meiotic recombination. Klar (1980) has shown that the formation of ascospores only requires the presence of duplicated spindle plaques for prospore wall to form on, whether these spindle plaques derive from a first meiotic ( see p.38), second meiotic, or mitotic division ( see p.42).

The ability of cells to sporulate in distilled water after three hours exposure to sporulation medium indicates that by this time they have synthesised all the necessary precursors for premeiotic DNA synthesis, and Simchen et al.(1972) have shown that premeiotic DNA synthesis differs from mitotic DNA synthesis in using internal pool precursors with no recycling.

Pinon (1977) has shown that the point of commitment to sporulation coincides with the end of the period of sensitivity to ammonia. In the early stages ammonia inhibits sporulation but does not affect commitment to premeiotic DNA synthesis and meiotic recombination.

## Cell Cycle Control - The Initiation of Sporulation

The initiation of sporulation depends on the stage reached by the cell in the mitotic cell cycle. Cells can only initiate sporulation in the G1 phase of the cell cycle (Haber & Halvorson, 1972) prior to mitotic DNA synthesis. The initiation of sporulation clearly must occur before commitment to mitosis, which occurs prior to DNA synthesis and which is dependent on the cdc 4 function (Hirschberg & Simchen, 1977). It is likely that by this time cells have undergone events essential for subsequent DNA synthesis without recombination necessary for meiosis (Hopper et al., 1975) and therefore cannot proceed to meiosis and sporulation.

The inter-relationship of life-cycle events has been studied closely using cdc (cell division cycle) mutants (Hartwell, 1970; Reed, 1980). These are temperature sensitive mutants which, at their/

/restrictive temperature, arrest at a characteristic point in the cell cycle, with a characteristic phenotype (the "terminal phenotype"). The expression of the wild type genes defined by the cdc mutations can be ordered temporally in a sequence in which some gene products are dependent on others and upon cell cycle events, and vice versa (Hartwell et al., 1973; Hereford & Hartwell, 1974). Some cdc mutations that arrest cells in the mitotic cell cycle also prevent sporulation (Simchen, 1974), indicating that some mitotic functions are also required in meiosis. Interestingly, some cdc mutations also prevent sporulation at the permissive temperature for growth, indicating that some mitotic functions are used, but with different mechanisms. The significance of this work is, however, not clear, as the restrictive temperature of most cdc mutants (36°C) is too high for sporulation to occur, and the intermediate temperature used (33.5°C) is close to the upper limit for sporulation, and also close to the lower limit of the restriction on cell division.

The cdc 25 and cdc 35 mutations appear to be in genes normally operating close to the sporulation initiation point since the mutations promoted sporulation on temperature shift-down even when cells carrying them were introduced into rich media (Shilo et al., 1978). Hartwell (1976) was able to show that DNA synthesis was dependent on some previous cdc functions, and others in turn were dependent upon DNA synthesis.

Related to the problem of spore initiation is that of the stationary phase since, for a diploid cell, it is normally the only developmental alternative to sporulation or vegetative growth. Cells starved of nutrients accumulate in the G1 phase of the cycle, taking the characteristic single, unbudded phenotype and normally rounding up and becoming phase-bright. This stationary phase was considered for a long time to be a prolonged G1 phase that the cells took up because they were unable to initiate any other process. However, recent evidence (Pinon, 1978) indicates that starved cells undergo a series of morphological (and presumably metabolic) adaptations to dormancy; this condition has been termed the Go phase. Stationary cells can/

/proceed to meiosis without a further cell cycle (Hirschberg & Simchen, 1977). The stage in the cell division cycle at which cells enter this Go condition appears to be just before the "start" event defined by the cdc 28 mutation (Reed, 1980; Bedard et al., 1981) which is considered to be the point at which mitosis is initiated. Cells in Go are distinguished by chromosome structure (Pinon, 1978) and staining properties (Swartzendruber, 1977).

Evidence will be presented in this thesis that there are mutations (spo 0) conferring asporogeny by affecting the initiation of sporulation that also prevent the cells entering a starvation-induced programme (i.e. a Go phase) without interfering with their ability to undergo G1 or other cell division cycle processes (see also Dawes et al., 1980; Dawes & Calvert, 1981; Calvert & Dawes, 1984)

Related Cell Cycle Events: G1 phase and Cell Size Controls

The initiation of sporulation is dependent on the cell division cycle since it can only occur in cells which have reached some point in the G1 phase (Hirschberg & Simchen, 1977). It is worth, therefore, discussing the controls on cell division which operate during this phase, since this will shed light on possible meiotic control mechanisms.

The cell division cycle in yeast can be divided into four phases: G1, in which the cell prepares to commence DNA synthesis and budding; S (synthetic) during which the cell replicates its DNA and the bud initiated at the start of S enlarges; G2 during which the cell completes bud growth and doubling of cell contents, and prepares for mitosis, and M, during which mitosis occurs, one daughter nucleus is packaged into the bud, and cytokinesis follows.

In a number of eukaryotic species, the time taken from the initiation of DNA synthesis through to mitosis (i.e. the S + G2 phase) is, for a given cell type, relatively constant over a wide/

/range of generation times. Longer generation times are due a almost entirely to an expanded G1 phase (For a review, see Mitchison, 1971). This observation, although derived from mammalian tissue culture, holds good for a number of cell types, including Saccharomyces cerevisiae under a variety of conditions (Slater et al., 1977; Carter & Jagadish, 1978; Johnston et al., 1980). In chemostat cultures growing at specific growth rates greater than $0.1h^{-1}$, the time from bud initiation to cell separation, which corresponds to the phases S + G2 + Mitosis (Slater et al., 1977) is independent of growth rate (Beck & von Meyenburg, 1968). The cell appears to continue growth in G1 until a trigger point at which mitotic preparation events commence, and this trigger event is known as 'start' (for a general review, see Pringle & Hartwell, (1981). By the use of cdc mutants, Hartwell & coworkers (Hartwell et al., 1970; Hartwell et al., 1973; Hartwell et al., 1974; Hartwell, 1974) were able to demonstrate the existence of a transition point in G1 that was specifically timed and mediated by the product of the CDC 28 gene. This transition point corresponded with the initiation of the cell cycle and was identified with the 'start' event. Cells arrested in G1 by nutrient starvation accumulate at a point prior to 'start' (Johnston et al., 1977). The escape from arrest at 'start' may be mediated by the CDC 28 gene function (Hartwell, 1974; Reed, 1980; Bedard et al., 1981) although this gene's product has been shown recently to function also later in the cell cycle prior to DNA synthesis and the areas of the gene responsible for the two functions are distinct (Rai et al., 1982). Cells arrested by a cdc 28 mutation show one of two terminal phenotypes according to the allele present: some arrest as unbudded oval cells while others fail to undergo nuclear division cycle events but continue growth, producing large, misshapen cells.

One of the first indications of a specific size control operating at 'start' came from work by Johnston and coworkers (Johnston et al., 1977b; Johnston, 1977) on the behaviour of yeast cells under nitrogen starvation. These cells continued division but degraded RNA and protein to recycle nitrogen, and/

/the average cell size became markedly smaller. The small cells produced, however, were first-generation daughter cells, and further budding occurred only in normal-sized cells. They suggested that there was a critical size which had to be reached by a cell before it could initiate budding. Further work (Carter & Jagadish, 1978; Johnston et al., 1979; Lorincz & Carter, 1979) showed that there was a uniform cell size at bud initiation which was medium dependent. Under given medium conditions, the cells grew in G1 to a critical size, after which budding commenced, and the time to mitosis was then constant.

A similar size control has been established in Schizosaccharomyces pombe (Fantes & Nurse, 1977) but in this case the main control operates at mitosis itself, although there is a subsidiary one that operates at the initiation of DNA synthesis (Nurse, 1975; Nurse & Thuriaux,1977). In this species, it was possible to isolate mutants, designated wee mutants, which initiated mitosis at a reduced size and were therefore altered in their size-control. (Thuriaux et al., 1978).

Size control mutants of Saccharomyces cerevisiae were mutant at either of two loci, whi 1 and whi 2 (Carter & Sudbery, 1980; Sudbery et al., 1980). Mutant strains initiated DNA synthesis at a small size and therefore proceeded to divide at a small size. Strains carrying whi 1 were reduced in size throughout growth, whereas strains carrying whi 2 were normal during exponential phase, but became reduced in size when they were nutrient limited. Neither type of strain was able to enter a normal stationary phase, and therefore viability was lost under starvation conditions.

A number of possible molecular mechanisms for the control of cell size have been suggested. Ycas et al. (1965) put forward a single inhibitor-dilution model in which the unstable inhibitor is produced at a constant rate proportional to the number of genome equivalents in the cell. This means that as the cell increases in size, the absolute intracellular concentration of the/

/inhibitor falls, until a critical level is reached, at which the cell is able to initiate the dependent morphological sequence. Fantes et al.(1975) suggested a number of possible mechanisms based on further evidence and investigated the characteristicss of their models using computer simulation. The size-control data appear to fit best to an inhibitor-dilution model as described above (Lorincz & Carter, 1979). The average size of Saccharomyces cerevisiae cells is dependent upon ploidy, as predicted by the model, but the volume of diploid cells is only 1.5-1.8 fold greater than that of haploid cells (Adams, 1977), not twofold as predicted.

Since 'start' is by definition the event initiating the cell cycle, it is evidently this event that is under size control. The temporal relationship between spore initiation and 'start' is not clear. Cells arrested before 'start' are able to initiate sporulation without undergoing an intervening cell cycle (Hirschberg & Simchen, 1977) indicating that spore initiation occurs after 'start', but it must be borne in mind that the conditions likely to cause a cell to undergo 'start' are liable to severely repress sporulation (See pp 90 and 102 ) making this interpretation unlikely. Shilo et al.(1978) also placed the initiation of sporulation after 'start' on the basis of the temporal ordering of the cdc 28 ('start') and cdc 25 and cdc 35 (Sporulation initiation) mutations. Recent evidence (Calvert & Dawes, 1984; and this thesis, p.94 ) indicates that spore initiation occurs before 'start'. Evidence is presented in this thesis that there is also a cell-size control over spore initiation.

## Genetic Control over Sporulation

Asporogenous mutants. The most obvious first step in the examination of genetic control over sporulation is the isolation of asporogenous mutants, and the characteristics of the mutations involved. This approach has two major problems. Firstly,/

/asporogenous mutations can only be detected in a diploid cell, as only these cells sporulate, but mutagenesis of diploid cells will yield mostly heterozygous strains in which recessive mutations will not be expressed. Mutation of heterothallic diploids will therefore yield only a small fraction of the mutations that can be isolated. Also, normal methods of genetic analysis in yeast involve mating haploid strains and sporulating the cross to yield recombined haploid meiotic products. This route is not possible in an unconditionally asporogenous mutant.

Esposito & Esposito (1969) used spores of homothallic strains to isolate recessive mutations in a homozygous conditions. If temperature sensitive mutants were isolated that sporulated normally at the permissive temperature, normal methods of genetic analysis could be used. Much of the subsequent work on genetic control has been done by the Espositos or using their mutations (For reviews, see Esposito & Esposito, 1975; Esposito & Esposito, 1978; Esposito & Klapholz, 1982)

By isolating and characterizing large numbers of spo mutations, assigning them into complementation groups, they estimated statistically that there were 48 ± 27 genes essential for sporulation and not cell division (Esposito & Esposito, 1972). Of the isolations made, three were dominant (SPD) mutations and therefore good candidates for mutations in regulatory genes (Esposito & Esposito, 1974b).

The time of expression of genes defined by temperature-sensitive mutations can be measured by temperature-shift experiments and the order of expression found. This has been done for the sporulation mutations spo1-1, spo2-1 and spo3-1 (Esposito et al., 1970). All three allow commencement but not completion of meiosis. Further work with these mutants has shown that the processes of prospore wall formation, growth and closure of spore walls, and nuclear budding are not dependent on each other, and can be separated by selective blocking with/

/particular mutations (Esposito et al.,1972; Moens et al., 1974)

The mutation spo3 causes a low level of production of two-spored asci, and genetic analysis of these shows that they are diploid products of a reductional (Meiosis I-like) division (Esposito et al., 1972) similar to those produced by heat shock of sporulating cells (Davidow et al., 1980). This demonstrates that the meiosis II division is not essential for packaging of viable spore nuclei. A surprising result was obtained by genetic analysis of the two-spored asci produced by spo12 and spo13 mutants (Klapholz & Esposito, 1980a; 1980b). In these mutants, the low levels of two spored asci were found to contain diploid products of an equational (meiosis II-like) division, indicating that a meiosis II-like division had occurred without a prior meiosis I. In strains carrying spo12 or spo13 and Rad 50-1, a Rec (Recombinationless) mutation, viable ascospores can be obtained without prior meiotic recombination normally required for sporulation (Esposito & Esposito, 1974a; Malone & Esposito, 1981).

Several problems exist in the use of general asporogenous mutants to study sporulation. The precise nature of the biochemical lesion underlying a blockage in sporulation is difficult to ascertain in a mutant which may fail to undergo several other events which are dependent on the one blocked. Also, very few sporulation-specific biochemical events are known, and therefore the lesions are difficult to test for. As will be discussed later (p.72 ) it is difficult to isolate initiation mutants in this way, as the probability of a general asporogenous mutant falling into this category is low. Also, this type of isolation will tend to miss two sorts of mutation: those which cannot be isolated as temperature sensitive, and those which do not arrest with a distinctive phenotype.

A number of other types of mutation have been shown to block sporulation (For review, see Esposito & Klapholz, 1981. Roth (1973) isolated three mutations, designated mei 1, mei 2 and mei 3, which blocked premeiotic DNA synthesis. In this case/

/carbohydrate, protein and RNA were still synthesized after the block. The production of proteases is important during sporulation due to the need to mobilize precursors for protein synthesis, and they are coordinately repressed by ammonia in sporulation (Opheim, 1979). The removal of proteases by mutation has varying effects on sporulation according to the extent of the reduction in proteolytic activity (Betz, 1979; Zubenko & Jones, 1981).

Nuclear petite mutants (PET)are unable to sporulate (Ephrussi & Hottinguer, 1952) although more MIT mitochondrial mutants with severely deficient respiration are able to sporulate (Pratje et al., 1979; Hartig & Breitenbach, 1980). In this case, as in most others, it is difficult to tell whether a mutation is involved in the genetic control of sporulation or whether it simply blocks a process essential to it.

A number of other mutations have been shown to block, or severely repress, sporulation, including Rad 50 and Rad 52 (but see p.38 ) shown by Prakash et al. (1980), Hk 1 which blocks response to catabolite repression (Stark et al., 1980) and some amino acid biosynthetic pathway mutations (Wejksnora & Haber, 1974).

Derepressed Mutations An alternative approach to the genetic control of sporulation is to isolate derepressed mutations, that is mutations causing cells to sporulate under conditions in which the wild-type does not, and therefore analagous to the constitutive mutations of the lac operon of E.coli (Jacob & Monod, 1961).

Derepressed sporulation mutants were first isolated by Dawes (1975) and the mutation in them designated spd 1. These mutants fail to grow vegetatively on non-fermentable carbon substrates, especially glycerol, and under these conditions diploids homozygous for spd 1 sporulate massively. Their efficient sporulation, and their ability to grow on ethanol as/

/sole carbon source, indicate that spd mutations are not nuclear petites. Three unlinked loci at which these mutations can occur have been identified, designated spd 1, spd 3 and spd 4 (Dawes & Calvert, 1984).

The spd 1 mutation is highly pleiotropic, causing derepressed sporulation of cells reaching stationary phase in glucose media, derepressed sporulation of cells growing on glycerol, G1 arrest of haploids growing on glycerol, and relative insensitivity to nitrogen inhibition of sporulation (Vezinhet et al., 1979).

Strains carrying spd 1 are able to take up glycerol (Kinnaird, 1979; Vezinhet et al., 1979) and evidence presented later in this thesis will show that they are not physiologically unable to utilize glycerol for growth, and also they are able to utilize it as a carbon and energy source for sporulation. Polyacrylamide gel electrophoretic analysis has shown several specific polypeptides that are derepressed in spd 1 strains (Dawes et al., 1980; Dawes & Calvert, 1984). It has also been established that the point at which spd 1 arrests haploid cells in G1 at or prior to, the execution point of the CDC 28 gene (Vezinhet et al., 1979).

The phenotype of spd 1 mutants is therefore exceedingly complex, but their characteristics are of great interest. Although they have the metabolic machinery to take up, and utilize glycerol they fail to do so, and sporulate massively. Although non-utilization of glycerol is normally a characteristic of respiratory deficient and therefore asporogenous mutants, these mutants hypersporulate. It is thus one of the few mutants in which a sporulation defect is directly linked to a physiological defect. They are of interest in establishing the mechanisms determining whether a cell sporulates or continues vegetative growth at the branch point in the G1 phase of the cell cycle,/

/since they are capable of both and their defect lies in the predisposition to one path, rather than simply a block in the other.

One of the interesting characteristics of the spd 1 mutants is the frequency with which they revert to growth on glycerol (Dawes, 1975; Calvert & Dawes, 1984), producing mostly asporogenous revertants. A later chapter in this thesis will describe more fully the characteristics of these asporogenous revertants.

Mating-Type Control As described previously, haploid yeast cells can be of a or α mating-type, according to the allele present at the mating-type locus (MAT), the two alleles being respectively MATa or MATα . MATa and MATα control a number of functions of the cell, such as production of mating pheromones, ability to sporulate, ability to respond to mating pheromones, zygote formation and conjugation. Mutations causing mating defects can be of two types; either mapping at the mating type locus itself, in which case they are designated mata or matα , or else mapping elsewhere, in which case they have a variety of designations (Mackay & Manney, 1974a; 1974b; Kassir & Simchen, 1976). The second class presumably affect genes involved in the processes controlled by MATa and MATα . Loss of ability to sporulate is characteristic of heterothallic diploids carrying mat a and mutations in the MAT α -2 region (Kassir & Simchen, 1976; Herskowitz & Oshima, 1981), indicating a requirement for information from MATa and MATα .

It has been known for some time that heterozygosity at the mating type locus is necessary for sporulation. Roman & Sands (1953) demonstrated that a/a and α/α diploids do not sporulate, although a/a/α/α tetraploids are able to. Homogygous a/a and α/α diploids fail to undergo a number of sporulation-specific processes, including premeiotic DNA synthesis (Roth & Lusnak, 1970; Hopper et al., 1974), intragenic recombination (Friis & Roman, 1968), glycogen breakdown, RNA degradation and protein degradation (Hopper et al., 1974)

A number of mutations have now been isolated which enable diploids homozygous at MAT to sporulate. The sca mutation of Gerlach (1974) is unlinked to MAT and allows both a/a and α/α cells to sporulate. The CSP1 mutation of Hopper et al. (1975) also allowed both a/a and α/α to sporulate and, further α/α CSP1 strains were indistinguishable from α/α ± in ability to produce mating pheromone, conjugate, form zygotes, undergo intragenic recombination, and in X-ray sensitivity and so CSP1 appears to be specific to sporulation control rather than other mating type control functions. The rme mutation enabled α/α cells to sporulate, but not a/a (Kassir & Simchen, 1976), and also enabled sporulation of MATa/mata and mata / MATα diploids, both of which are normally unable to sporulate. Similarly, the SAD1 mutation (Hopper & Mackay, 1980; Kassir & Herskowitz, 1980) allowed sporulation of MATα /MATα , MATα/mata-1 and MATα/matα-2 but not MATa/MATa or MATa/o (monosomic for chromosome III) diploids.

Strathern et al. (1981) proposed that two controlling functions were exercised by the MATα gene, via the MATα-1 gene product which acts both as a negative regulator of a-specific functions and a regulator of a/α functions. Herskowitz & Oshima (1981) modified this to suggest that the combination of MATa and MATα-2 gene products is the controlling element in sporulation. By this model, a matα-1 matα-2 haploid should therefore be unable to switch on α functions or switch off a functions, and should mate as an a, but the resulting matα-1 matα-2/MATα triploid should be an α mater, and unable to sporulate, since it lacks the MATa product. These predictions have been confirmed. (For a review, see Herskowitz & Oshima, 1981).

Interesting work by Klar (1980) has demonstrated that the mating type regulators operate via the cytoplasm. The kar 1 (karyogamy-deficient) mutation prevents nuclear fusion after mating. If an α/α strain is mated to an a karl strain, the resultant triploid remains as a heterokaryon with one/

/diploid and one haploid nucleus. The heterokaryon sporulates to produce six spores, and genetic analysis shows that four of the spores are derived from a meiotic division of the $\alpha/\alpha$ nucleus, and two of the spores are derived from a mitotic division of the a kar 1 nucleus. This has several implications: firstly that the MAT$a$ and MAT$\alpha$-2 products can combine to promote sporulation despite being produced by different nuclei, second that a MAT$\alpha$/MAT$\alpha$ nucleus is capable of undergoing meiotic division and incorporation into spores, and third, that prospore wall can form on a mitotic spindle pole body and a mitotically divided nucleus can therefore become a spore nucleus.

## Aims of the Present Work

The aims of this present work were as follows:

(i) To further investigate the physiology and genetics of spd 1 mutants, including their response to various external media and their interaction with other initiation mutants, including any linkage of the mutations responsible; and also to quantify their high rate of reversion to growth on glycerol.

(ii) To characterize the revertants thus obtained, to isolate any second site suppressor mutations and establish the number of genes involved and their linkage to other mutations involved in initiation. By this method it was hoped that mutants specifically defective in initiation of sporulation could be isolated and used to examine the relationship between nutrient conditions and the cell's ability to sporulate. The mutants obtained also throw some light on the processes occurring in stationary phase in yeast.

(iii) To find, and characterize, any size control over sporulation similar to that over cell division, by using the properties of yeast growing in continuous culture.

(iv) To use the continuous culture method to examine nutrient control over sporulation by inducing yeast cells to sporulate at a continuous, steady rate in a chemostat, and varying the nutrient stress, both for wild-type cells and spd 1 mutants.

(v) To detect any new gene expression during sporulation by extracting mRNA and using an in vitro translation technique to detect the corresponding proteins and thus detect any new messengers produced.

# CHAPTER 2

## MATERIALS AND METHODS

## STRAINS USED

| STRAIN | SOURCE | GENOTYPE | NOTE |
|---|---|---|---|
| S41 | I W Dawes Culture Collection | $\frac{MAT\,a\ HO\ HML\alpha HMR\,a}{MAT\,\alpha\ HO\ HML\alpha HMR\,a}$ $\frac{arg4\text{-}1\ \ cyh\ 1}{arg4\text{-}1\ \ cyh\ 1}$ | |
| 124-8A | " | $\frac{MAT\,a\ HO\ HML\alpha HMR\,a}{MAT\,\alpha\ HO\ HML\alpha HMR\,a}$ $\frac{ura\ 1}{ura\ 1}$ | |
| 59-4A | " | $\frac{MAT\,a\ HO\ HML\alpha HMR\,a}{MAT\,\alpha\ HO\ HML\alpha HMR\,a}$ $\frac{arg4\text{-}1\ cyh\ 1\ spd1\text{-}1}{arg4\text{-}1\ cyh\ 1\ spd1\text{-}1}$ | (1) |
| 95 | " | $\frac{MAT\,a\ HO\ HML\alpha HMR\,a}{MAT\,\alpha\ HO\ HML\alpha HMR\,a}$ $\frac{ura\ 1\ spd\ 3}{ura\ 1\ spd\ 3}$ | |
| 69-10C | " | $\frac{MAT\,a\ HO\ HML\alpha HMR\,a}{MAT\,\alpha\ HO\ HML\alpha HMR\,a}$ $\frac{arg\ 4\text{-}1\ spo50}{arg\ 4\text{-}1\ spo50}$ | (2) |
| 39 | " | MATa his4 | |
| 24 | " | MATa met5 trp1-1 | |
| 25 | " | MAT$\alpha$ met5 leu2-1 | |
| 129 | " | MAT$\alpha$ met1 | |
| ST34 | S I REED | MATa cdc28-15 tyr1 lys2 cyh1 | (3) |

| STRAIN | SOURCE | GENOTYPE | NOTE |
|---|---|---|---|
| BR207-7a | J R PRINGLE | MATa cdc25-3 adel/2 ural trpl his7 arg4 isol | (4) |
| BR220-169α | J R PRINGLE | MATα cdc35-4 adel/2 ural trpl arg4 | (5) |
| SU23 | P SUDBERY | MATa lys2 whil | (6) |
| SU109 | P SUDBERY | MATα met whi2 | (6) |
| 168-2D | | MATα spdl-1 metl | (7) |
| 168-2A | | MATa arg4-1 | (7) |
| 206-1B | | $\frac{\text{MAT}_a\ \text{HO}\ \text{HML}_\alpha\text{HMR}_a}{\text{MAT}_\alpha\ \text{HO}\ \text{HML}_\alpha\text{HMR}_a}$ leu2-1 spdl-1 / leu2-1 spdl-1 | (8) |
| 248-4C | | $\frac{\text{MAT}_a\ \text{HO}\ \text{HML}_\alpha\text{HMR}_a}{\text{MAT}_\alpha\ \text{HO}\ \text{HML}_\alpha\text{HMR}_a}$ ural whil / ural whil | (9) |
| 249-4A | | $\frac{\text{MAT}_a\ \text{HO}\ \text{HML}_\alpha\text{HMR}_a}{\text{MAT}_\alpha\ \text{HO}\ \text{HML}_\alpha\text{HMR}_a}$ metl his4 whi2 / metl his4 whi2 | (10) |

NOTES

(1) The spdl-1 mutation is the strongest spd allele.
(2) The first spo0 strain - derived from 594A.
(3) Reed (1980).
(4) Shilo et al. (1978)
(5) Shilo et al. (1978)
(6) Sudbery et al. (1980), whil and whi2 are mutations affecting cell size control.
(7) Segregants from cross of 129 x 59-4A.
(8) Segregant from cross of 25 x 59-4A.
(9) Segregant from cross of SU23 x 124-8A.
(10) Segregant from cross of SU109 x 124-8A.

GROWTH AND SPORULATION MEDIA

Strains were routinely maintained on YEPD agar containing, per litre, 10g yeast extract, 20g peptone, 20g glucose, 20g agar (Oxoid number 3), 0.1g adenine and 0.1g uracil (Sigma). Liquid YEPD medium contained no agar. Other complex media used were YEPG, containing 30ml glycerol as carbon and energy source in place of glucose. Similarly YEPA contained 20g potassium acetate in place of glucose. YEPGAL contained 20g galactose in place of glucose.

The basic minimal medium used contained per litre, 1.6g Difco yeast nitrogen base (without amino acids or ammonium sulphate), 5g ammonium sulphate, 20g glucose and 20g agar if desired, and was adjusted to pH7. Amino acids were supplemented as required at the following concentrations per litre; threonine 0.3g; lysine 0.04g; leucine 0.03g; tryptophan, methionine, histidine, arginine, adenine, uracil, 0.02g; tyrosine, phenylalanine 0.003g.

GALMIN contained galactose in place of glucose in the minimal medium formula.

GASP (glycerol aspartate) medium contained glycerol at 30ml $l^{-1}$ in place of glucose, aspartate at $2gl^{-1}$ and the full set of amino acids and nucleotides as above.

Cells were induced to sporulate in liquid media by re-suspending in potassium acetate solution ($20gl^{-1}$, adjusted to pH7). For sporulation on solid media, this was supplemented with $20gl^{-1}$ agar number3 (Oxoid), $2.2gl^{-1}$ yeast extract, and $0.6gl^{-1}$ glucose.

Three media were used in chemostat studies:-
A) A nitrogen-limited medium containing $1.5gl^{-1}$ difco yeast nitrogen base, $0.1gl^{-1}$ ammonium sulphate, $0.05gl^{-1}$ arginine, $0.55gl^{-1}$ potassium dihydrogen orthophosphate, $1gl^{-1}$ di-potassium hydrogen orthophosphate, and $10gl^{-1}$ galactose.
B)/

B) A galactose limited medium containing $1.5gl^{-1}$ Difco yeast nitrogen base, $2gl^{-1}$ ammonium sulphate, $0.05gl^{-1}$ arginine, $0.55gl^{-1}$ potassium dihydrogen orthophosphate, $1gl^{-1}$ di-potassium hydrogen orthophosphate and $1gl^{-1}$ galactose.

C) Galactose limited medium containing $1.5gl^{-1}$ Difco yeast nitrogen base, $5gl^{-1}$ ammonium sulphate, $1gl^{-1}$ yeast extract, $0.55gl^{-1}$ potassium dihydrogen orthophosphate, $1gl^{-1}$ dipotassium hydrogen orthophosphate, $10gl^{-1}$ galactose, and 0.1g of each of the 11 supplementary amino acids and nucleotides as listed above.

## GENETIC ANALYSIS

Genetic analysis was by standard techniques (Sherman, 1975; Mortimer & Hawthorne, 1969). Strains to be mated were chosen with complementary auxotrophic markers, and were mixed, allowed to mate, and replica plated onto minimal medium to isolate mated clones. To assess linkage, mated strains were allowed to sporulate on potassium acetate agar (to obtain meiotic recombination) and asci were then treated with glusulase (sigma) to digest ascus walls. Individual asci were picked out and dissection performed on a Leitz micromanipulator, separating individual spores to obtain clones (Sherman, 1975). Auxotrophic markers were detected by replica plating cultures onto drop-out media (minimal media supplemented with all but one of the supplementary amino acids and nucleotides listed above.

### Measurement of Growth Yields and Percentage Ascus Formation

To assess the ability of yeast strains to grow and sporulate using particular carbon sources, media were made up containing $10gl^{-1}$ yeast extract, $20gl^{-1}$ peptone and $10gl^{-1}$ of the test carbon substrate (sodium salt where appropriate). Strains were inoculated from solid media into 100ml volumes of test medium and the culture was incubated at $30^{o}C$ with shaking, for 40h. After this time, the absorbances of the culture at 600mm were measured, as an estimate of the growth yield, and the percentage of asci among the cells in each culture was measured by microscopic examination under phase-contrast illumination at x40 magnification.

Oxygen Consumption

Rates of oxygen consumption by strains were measured using a Warburg manometer, inoculating cells into a scaled flask connected to the manometer, absorbing gaseous carbon dioxide in a well of potassium hydroxide, and measuring the reduction in volume of the gas above the culture due to oxygen uptake.

Growth of Cells on Media Preincubated with Cross-Feeding Organisms

Liquid YEPG and YEPA media were inoculated with the bacterial cross-feeding organism and incubated for 24h at 30°C with shaking. The cells were removed by centrifugation at 10,000g for 15min and the spent medium was decanted and reautoclaved. Cells of strains S41 and 59-4A were inoculated into 100ml volumes of spent medium and unused medium, and the cultures were incubated at 30°C, with shaking, for 24h. The absorbance of the cultures at 600mm were then measured.

Reversion Rate of spdl Strains to Growth on Glycerol

A culture of 59-4A was streaked out on YEPD for single colonies and after incubation, a single clone was inoculated into YEPD liquid medium, ensuring that the clone was 'young' so that there was no selective pressure for revertants. During the exponential phase of growth, a sample was taken, serial dilutions were made on YEP liquid medium, and appropriately diluted suspensions were spread on YEPD plates for total viable counts, and onto GASP medium to detect revertants. Similarly, a culture of S41 was plated onto GASP medium. After four days incubation, small colonies of S41 were visible on the GASP plate, and at this time the numbers of papillae on the plates of 59-4A were measured. The minimum time for colony formation was used in this way in order to ensure that the papillae counted were due to 'revertants' already present in the culture, and not those selected during incubation on the GASP medium.

## Isolation of spo0 mutations

### Isolation from diploid spd1 strains

In order to isolate 'revertants' from diploid spd strains, they were streaked out on YEPD agar and then replica-plated onto YEPG agar. After about 2 weeks incubation at 30°C, numerous small papillae appeared. Each clone was picked off and streaked out for pure culture on YEPG agar. The clones were tested for their ability to sporulate, and those that sporulated were dissected and the segregants characterised.

### Isolation from a haploid spd1 strain

'Revertant' clones were obtained from the haploid spd1 strain 168-2D as described above. The 'revertants' obtained were backcrossed to the wild-type strain 168-2A and the resultant cross dissected. In those cases where the spd1-1 mutation was recovered, and in which, therefore, the reversion was due to a second-site mutation, clones containing only the revertant mutation were isolated (as detected by inspecting the segregation in individual asci). Clones of complementary genotype were mated and the resultant diploids were plated onto KAc medium to detect whether the mutation conferred asporogeny when in homozygous state.

### Loss of Viability of spo0 strains under starvation conditions

Four strains, S41(wild type), 69-10C(spo50), 246-1B(spo51), and 247-2D(spo53) were inoculated into 100ml volumes of YEPA and incubated at 30°C with shaking. Viable counts were taken at the point of maximum turbidity (as measured by the absorbance at 600mm), and thereafter at various times up to 5 days after starvation had occurred.

### Photomicrography of Yeast Cells

In order to obtain the photomicrographs of yeast cells/

/comprising figures 4.6 to 4.9, the strains were incubated in YEPA liquid medium until starvation conditions were reached. After 24hr, samples were taken and a single drop of each placed on a clean microscope slide. A drop of 50% glycerol was placed on the slide and the two drops mixed. (The glycerol was added to reduce mobility of the cells). Photomicrographs were taken under phase contrast illumination at x90 magnification on a Leitz microscope with matching photographic attachments.

Suppression of spd mutations by spo0 mutations

Homothallic diploid strains containing each of the three spo0 mutations were inoculated onto KAc sporulation agar and incubated at 30°C for seven days to obtain a few spores. The strains were then mated to spores of strains 59-4A and 95, carrying spd1 and spd3 respectively, and the resulting crosses were dissected to examine the segregation of glycerol growth and non-growth characteristics.

Interactions between spd and whi Mutations

Five homothallic diploid strains were constructed which were (i) homozygous for whi1, (ii) homozygous for both whi1 and spd1, (iii) homozygous for whi2, (iv) homozygous for whi2 and spd1, (v) homozygous for spd1 only. Each strain was inoculated into YEPGal liquid medium and incubated at 30°C with shaking. After the cultures had entered exponential phase, a sample of each was taken and examined microscopically. A distribution of cell lengths was obtained by microscopic observation of 100-200 cells, measuring the length of each under x40 magnification using an eye-piece graticule.

Another sample was taken and the cells were harvested by centrifugation at 10,000g for 15min, washed and resuspended in KAc sporulation medium and incubated at 30°C for 48h. After the original YEPGal cultures had been in stationary phase for 12h, further samples were taken and the above processes were repeated. After the KAc suspensions had been incubated for 48h, the distribution of cell lengths was measured microscopically, and the percentage of asci in each culture was measured by microscopic examination.

## Chemostat Operation

All continuous culture experiments were performed using the apparatus shown in Figure 2.1. The chemostat used was a New Brunswick BIOFLO C-30 bench top fermentor with 305ml working volume culture vessel. Temperature was maintained at 30°C by a thermistor controlled heating element coupled with "cold-finger" cooling. Samples were taken direct from the culture vessel via a hooded sampler, discarding the first sample containing material from the sample-line dead space. Medium was fed into the culture vessel at a controlled rate via a Pharmacia P-1 peristaltic pump.

To operate, sterile medium was fed into the culture vessel and brought to 30°C with agitation at 800rpm and aeration with water-saturated air at 1-2l$min^{-1}$. The inoculum was added from a batch culture using a sterile syringe, inserting the needle through a rubber membrane over the inoculation port. Once inoculated, the culture was grown for 1 day without dilution, to adapt cells to chemostat conditions, and then the fresh medium flow was started, and the cells adapted to the new dilution rate. Samples were taken at least twice daily, and their turbidity, pH, ammonia levels, cell budding rate, cell sporulation rate and other parameters assessed.

## Measurement of Culture Parameters in the Chemostat

### Culture Turbidity

Culture turbidity was assessed by measuring the absorbance of the culture at 600nm.

### Percentage of Budded and Sporulating Cells

The percentage of budded cells and asci in each sample was assessed by microscopic examination at x40 magnification. In each case, a total of at least 1000 cells was examined and the proportion of budded cells and asci among these was assessed.

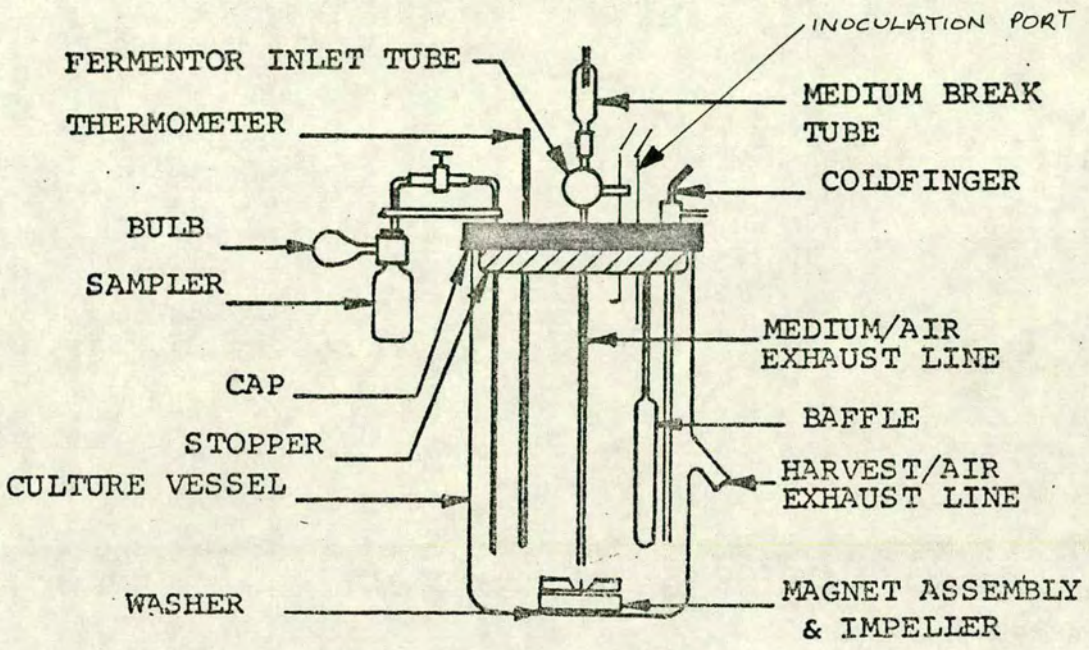


Figure 2.1 Configuration of chemostat for continuous cultivation of yeast.

Cell size

Cell lengths and volumes were measured under microscopic examination, using an eyepiece graticule which had been previously calibrated against a slide micrometer grating at each of the magnifications used. Cell length was measured at x40 magnification, revolving the graticule to measure the major diameter of each cell. Cell volumes were measured at x100 magnification, again revolving the graticule, but in this case measuring both major (a) and minor (b) diameters of the cell. Volume of the cells is given by:

$$V = \frac{\pi ab^2}{6}$$ (Wheals, 1982)

Ammonium Assays

Ammonia levels were not measured in a strictly quantitative manner. 3ml of each sample was centrifuged in a bench microcentrifuge to remove the cells, and the supernatant was decanted off. A single drop of Nessler's reagent was added to the supernatant, and if the sample showed neither a precipitate nor a colour change to yellow/orange, it was assumed that no detectable ammonia was present, and that since the carbon source was in excess, that ammonia had become limiting.

Extraction of RNA from Yeast

Yeast cells of the desired strain were incubated in 1l volumes of YEPAD medium (YEPA supplemented with 0.05 to (w/v) glucose), until a turbidity of 0.7 at 600mm was reached. The cells from each 1l volume were harvested by sterile centrifugation at 10,000g for 5 min, and resuspended in 200ml KAc sporulation medium. The suspension was incubated at 30°C for 24h. Immediately after resuspension, and at 2h intervals afterwards, 40ml samples were taken from the suspension, and immediately poured onto ice at -20°C, centrifuged at 3000g for 5 minutes and resuspended in the least necessary volume of 0.1M sodium acetate. The suspension was then frozen at -80°C.

To extract the RNA, samples were thawed, made up to 5ml with 0.1M sodium acetate pH5, containing 5mM EDTA, and broken with glass beads in a Braun homogenizer, adding diethylpyrocarbonate to 0.1% (v/v) just prior to breaking.

The suspension was removed, adjusted to 1% SDS and 2vol. water-saturated phenol added. This mixture was shaken at 60°C for 3 min, and then shaken on ice for 10min. The mixture was then centrifuged for 15min at 4000g and the aqueous phase removed. The aqueous RNA solution thus obtained was reextracted with 1 vol. phenol and the process repeated. The final aqueous RNA layer was removed, and the RNA precipitated with 2 vol ethanol at -20°C overnight. The precipitate was centrifuged down in a bench centrifuge, redissolved in 5ml distilled water, and 5ml 0.2M sodium acetate pH5 added. The RNA was then reprecipitated with 20ml ethanol at -20°C overnight. The process was repeated until the RNA had been reprecipitated at least three times, and an absorbance spectrum was run to assess purity. If this was satisfactory, the RNA was stored for further use as a precipitate under ethanol.

## Preparation of Rabbit Reticulocyte Lysate

(The method given is a modification of that of Pelham & Jackson (1976).)

### Preparation of Rabbits

Young 5-7lb white rabbits were injected subcutaneously with 3ml, 2.5ml and 2.0ml of 1.25%(w/v) acetylphenylhydrazine, on days 1, 2, 3 and 4 respectively of the 8-day preparation programme.

### Bleeding of Rabbits

This took place on the 8th day of the programme. 2000 units of heparin were made up to 2ml with nembutal in a sterile syringe, and injected into each rabbit via the ear vein. As soon as the rabbit was fully anaesthetized, a sterile needle was inserted through the chest wall into a ventricle of the heart, and about 80ml of blood withdrawn in a sterile syringe. The blood was immediately diluted by adding to 100ml ice-cold sterile isotonic saline (0.134M NaCl, 7-5mM $MgCL_2$, SmMKCL). A further 3ml nembutal were injected into the rabbit, causing rapid death.

## Lysate Preparation

The blood suspension was centrifuged at 3500g for 10min. The supernatant was removed with a vacuum aspirator, and the pellet washed three times in ice-cold isotonic saline to ensure removal of Heparin, a potent protein synthesis inhibitor. The volume of the packed cells was measured, and 1.5 volumes ice-cold distilled water were added, and the mixture well shaken. The lysate was centrifuged at 70000g for 15min, and the clear supernatant was put into 1ml aliquots and stored frozen at -80$^{o}$C.

## Reticulocyte Lysate Incubation and Assay

6mg of haemin (Sigma) were dissolved in the least possible amount of 5M potassium hydroxide and made up to 10ml with sterile distilled water. A 1ml aliquot of lysate was thawed, and 25ml of haemin solution added just before thawing was complete. 0.5ml of lysate was removed and the rest re-frozen. To 0.5ml of thawed lysate were added the following:-

| | |
|---|---|
| 30ml salts solution | (M potassium acetate<br>10mM magnesium acetate<br>100mM dithiothreitol) |
| 30ml CP solution | (0.2M creatine phosphate<br>0.52M Hepes ph7.4) |
| 12.5ml spermidine | (10mM) |
| 10ml micrococcal nuclease | (1mgml$^{-1}$ in distilled water) |
| 6ml calcium chloride | (0.1M) |
| 2.1ml creatine phosphokinase | (12mgmg$^{-1}$ in 50% v/v glycerol) |

The mixture was then incubated at 20$^{o}$C for 15 minutes to allow full digestion of endogenous messenger RNA. 25ml of 0.1M EGTA was then added to inactivate the nuclease by sequestering $Ca^{2+}$ ions.

The treated lysate was put into 30-100ml aliquots, as required, and yeast message and labelled amino acid were added, the concentrations used depending on the calculated optimum for the particular mRNA, and the desired activity in the final products. Immediately after addition of labelled amino acid, a zero time sample of 5 or 10ml was taken, and added to 0.8ml ice-cold distilled water. 0.4ml of M sodium hydroxide containing 0.5M hydrogen peroxide was added, the mixture was mixed well and left to stand on ice for 20min. After this time, 0.8ml of 25%(w/v) trichloroacetic acid was added to precipitate all of the protein. Further samples were taken from the lysate incubation at suitable intervals. TCA-precipated samples were vacuum filtered on Whatman GF/A glass-fibre filters and washed three times with 30ml ice-cold 10% (w/v) TCA. Filter papers were thoroughly dried in a hot oven.

Filter papers were placed in scintillation vials and covered with an appropriate volume (normally 3.5ml) of toluene/PPO/POPOP scintillation fluid, (toluene containing 0.4% (w/v) PPO, 0.05% (w/v) POPOP). Scintillation activity was measured in a Packard scintillation counter.

## Polyacrylamide Gel Electrophoresis

After incubation with RNA, samples of lysate carrying 100,000 counts.min$^{-1}$ incorporated activity were boiled for 1min with 0.25vol. of 5x sample buffer (0.02M Tris/MCL pH6-8, 2% SDS, 0.2% B-mercaptoethanol and 20% glycerol).

A 10%(w/v) acrylamide solution was prepared with 5.3ml 30% (w/v) acrylamide (29.8 acrylamide = 0.2 bisacrylamide), 4ml Tris/HCL pH 8-8, 6.7ml distilled water, and 20ml TEMED. A 20% (w/v) acrylamide solution was prepared, with 10.6ml 30% (w/v) acrylamide, 4ml buffer, 1.4ml water, and 20ml TEMED. The two solutions were placed in appropriate wells of a gradient maker, 20ml ammonium persulphate was added to the 10% solution, and 35ml to the 20% solution, and the solutions were run into a gel cassette, to form a 10-20% gradient gel. This was overlayed with water-saturated/

/n-butanol while setting. When set, the butanol was removed, and 4.5ml of a stacking gel solution, containing 2ml 30% (w/v) acrylamide, 4ml Tris/HCL pH6-8, 10ml $H_2O$, 20ml TEMED and 30ml ammonium persulphate, were added, forming sample wells with a 'comb' before setting.

To each sample was added 0.05vol of 1% (w/v) bromophenol blue, and the samples were loaded on top of the gel. Samples were electrophoresed at 7.5mA (constant current) until they reached the stacking gel/resolving gel interface, and were then electrophoresed through the resolving gel at 15mA.

## Scintillation Counting of Gels

Gels prepared as above were stained with Coomassie blue by immersion for 1-2hr in a 0.25%(w/v) solution of Coomassie Brilliant Blue in 45%(v/v) methanol, 9.2%(v/v) glacial acetic acid. Gels were destained in methanol/acetic acid solution (5% (v/v) methanol, 7.5% (v/v) glacial acetic acid). Gels thus stained were cut into strips corresponding to the 'tracks' of the samples, and the strips were placed in a continuous absorbance monitor and the absorbance at 280nm measured along the strip. This done, the strips were cut into 0.5mm slices perpendicular to the running direction. Each slice was placed in a scintillation vial and 0.5ml 30% hydrogen peroxide added. The vials were incubated for 3h at 55°C and 1h at 100°C, after which was added 5ml triton-toluene scintillant (66% toluene, 33% triton x-100, 0.4% (w/v) PPO, 0.05% (w/v) POPOP) and the scintillation measured in a Packard scintillation counter.

## Autofluorography of Gels

Complete gels were fixed in 20% (v/v) acetic acid overnight. The acetic acid was decanted, and the gel was dehydrated in DMSO for 1h. The DMSO was then replaced with fresh DMSO and the gel left for a further hour. This was then decanted and the gel was impregnated with PPO by immersing it in a solution of 20% (w/v) PPO in DMSO for 1h. The DMSO/PPO was decanted and replaced with/

/20% (v/v) acetic acid containing 5% (v/v) glycerol, and the gel left overnight. The gel was then heat/vacuum dried onto Whatman 3MM filter paper, clamped to KODAK XR1 x-ray film and exposed at -80$^{o}$C in the dark for an appropriate time. The film was developed for 4min in KODAK DX80 developer and fixed for 4min in KODAK FX80 fixer.

# CHAPTER 3 : THE PHYSIOLOGY OF MUTANTS DEREPRESSED FOR SPORULATION

## Introduction

The basic aims of the work presented here were to identify genes affecting the initiation of sporulation; by examining these to extend the understanding of the physiology of the process; and ultimately to try to trace the primary defects in the initiation mutants obtained. One class of such mutants that has already been identified comprises the spd (derepressed sporulation) mutants (Dawes, 1975; Vezinhet et al., 1979; Dawes & Calvert, in preparation; see also p.39 this thesis). These mutants are extremely useful in the study of areas of metabolism involved in initiation, as they combine the derepressed sporulation characteristic with a defect in respiratory metabolism (Dawes, 1975). They are also useful as parent strains for the isolation of further initiation mutants. This chapter reports preliminary studies characterizing important aspects of the physiology of the spdl mutants, while subsequent chapters are concerned more with the isolation and characterization of a different type of initiation mutant isolated using the derepressed mutant as parent.

In this chapter, the relationship between the growth characteristics of derepressed sporulation (spdl) mutants and their derepressed sporulation was examined more closely, to analyze how metabolic activity may influence sporulation in a wild-type cell. In particular, the different substrates on which the mutant is derepressed have been related to the way in which they are utilized, the respiratory capability of the cell, and its capacity to sporulate. The influence of the genetic complement of the cell has been studied by isolating mutations restoring to wild-type the growth characteristics of the spdl mutants, and characterizing their effect on sporulation. The frequency of such "reversion"* events has been examined here as it is a characteristic of the spdl mutant, but the specific characteristics of the "revertants" obtained will be examined in chapter 4.

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* NB Use of the terms "revertant" and "reversion" in regard to these mutations does not imply that they involve a restoration of the wild-type allele at the spdl locus.

The Extent of Derepression in spd1 Mutants

Table 3.1 compares the abilities of the wild-type strain S41 and the spd1-1 carrying strain S9.4A to grow and/or sporulate on a range of carbon and energy sources, using as measures the final culture turbidity and percentage sporulation of several cultures after 40h incubation.

On the two fermentable carbon sources, glucose and galactose (Suomalainen and Oura, 1971) both S41 and S9.4A cells were able to grow, and did not sporulate. Four carbon sources, glycerol, acetate, lactate and pyruvate, supported growth of the wild-type but not of the mutant, and the latter sporulated extensively. None of the other six substrates supported growth either in the wild-type or the

| CARBON SOURCE | S41 | | S9.4A | |
|---|---|---|---|---|
| | Percentage Sporulation | Final A 600 | Percentage Sporulation | Final A 600 |
| Glucose | 0 | 12.0 | 0 | 11.2 |
| Galactose | 0 | 15.2 | 0 | 16.4 |
| Glycerol | 1 | 2.6 | 80 | 0.6 |
| Acetate | 0 | 2.1 | 90 | 0.8 |
| Lactate | 0 | 1.9 | 80 | 0.4 |
| Pyruvate | 0 | 4.8 | 90 | 0.4 |
| Glutamate | 20 | 0.3 | 70 | 0.2 |
| Aspartate | 5 | 0.6 | 20 | 0.6 |
| Oxaloacetate | 0 | 0.3 | 60 | 0.2 |
| Succinate | 10 | 0.3 | 90 | 0.2 |
| ∝Ketoglutarate | 50 | 0.4 | 50 | 0.2 |
| Malate | 5 | 0.4 | 5 | 0.2 |
| NONE | 10 | 0.3 | 50 | 0.5 |

Table 3.1 Growth yield (measured by final A 600 after growth) and axis formation of wild-type diploid (S41) and an spd1 derepressed strain (S9.4A) on different carbon sources. Cultures were incubated for 40h at 30°C in liquid media containing $10gl^{-1}$ yeast extract, $20gl^{-1}$ peptone, and $10gl^{-1}$ of the relevant carbon source (as sodium salt where appropriate).

/mutant, and so no conclusion can be drawn from this.

The phenotype of derepressed sporulation in the spd1-1 mutant is not, therefore, limited to glycerol-grown cultures, but is expressed on several non-fermentable carbon and energy sources. This supports the conclusion of Vezinhet et al. (1979) that the spd1 mutants are defective in some central metabolic function associated with respiratory metabolism.

Source of Residual Growth on YEPG

It was observed that spd1-1 mutants underwent a certain amount of growth on YEPG medium, to an A600 of 0.7-0.8 units. The source of this residual growth was examined to determine whether the cells were using glycerol or other substrates in the yeast extract/peptone mixture. Figure 3.2 shows the effect of raising the glycerol concentration in YEPG on the final A600 at which S41 and 59.4A cease growth. It can be seen that S41 responds to higher glycerol concentrations with a correspondingly higher growth yield, up to a maxiumum growth yield, after which increased glycerol concentrations become slightly inhibitory. 59.4A, on the other hand, shows very little response to increased glycerol concentration.

In a different experiment (see Table 3.2) 59.4A cells were inoculated into nine different media, with various concentrations of yeast extract, peptone and glycerol. The final A600 after 48h incubation was measured for each culture. In each case, increasing the yeast extract and peptone concentrations together produced a marked increase in the final A600 which the strains were able to reach, while increasing the glycerol concentration did not.

The substrate that was utilized in residual growth on YEPG was therefore probably derived from the yeast extract/peptone mixture, and not from the glycerol. This indicated that the cells growing in YEPG were growing on small amounts of (probably fermentable) material in the yeast extract or peptone. These results indicated that the spd1 mutant was unable to use glycerol for vegetative growth.

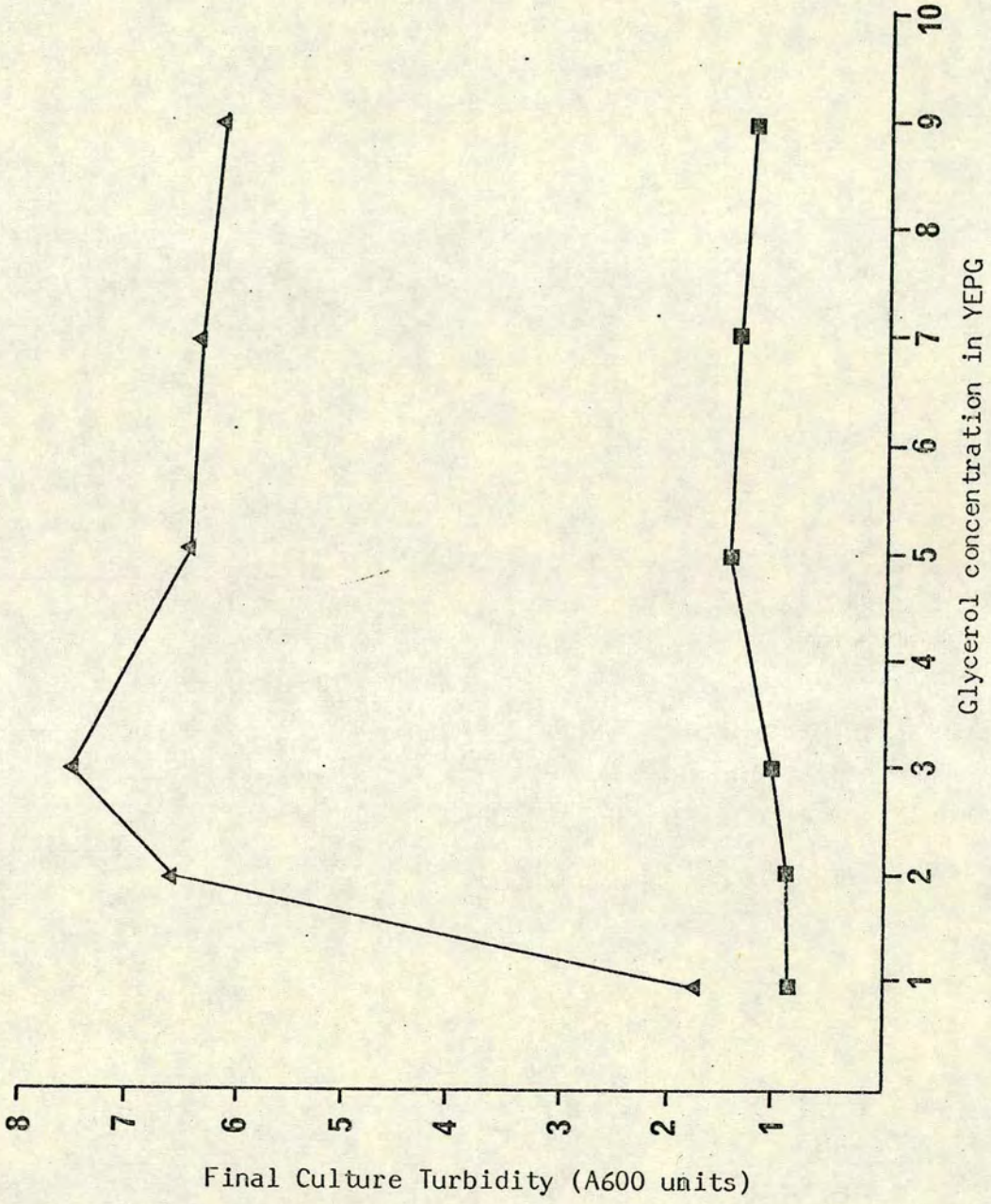


Figure 3.1 Effect of increasing glycerol concentration on the final culture turbidity reached by S41 (▲) and S9-4A (■) cultures. Cells were incubated at 30°C in liquid media containing 10gl yeast extract, 20gl peptone, and glycerol at the relevant concentration. The A600 was measured after 48h incubation.

| GLYCEROL CONCENTRATION (v/v) | RELATIVE STRENGTH OF YEP (NORMAL= 1%(w/v) Yeast Extract, 2%(w/v) Peptone) ½ x | 1 x | 2 x |
|---|---|---|---|
| 1.5% | 0.608 | 1.002 | 1.406 |
| 3% | 0.623 | 1.016 | 1.364 |
| 6% | 0.422 | 0.717 | 0.985 |

Table 3.2 The effect of different concentrations of YEP and glycerol on the final A600 reached by 59.4A cultures. Cells were incubated at 30°C in nine different media and the A600 of the cultures measured after 48h.

## Oxygen Consumption in spdl Strains

The rate of oxygen consumption by a culture is an indication of the activity of the TCA cycle in the cells and this is an important aspect to investigate in the spd mutants in order to trace their primary defect. The derepressing substrate used in this experiment was YEPA. It has been shown (Gosling & Duggan, 1969, 1971) that oxygen consumption is an important parameter in establishing the relative extent of TCA and glyoxylate cycle activities in the metabolism of substrates such as acetate and pyruvate.

During growth in the derepressing medium YEPA, and before the onset of sporulation, the oxygen uptake rates for S41 (wild-type diploid) and 59.4A (spdl-1 diploid) were measured at 1.8 ml.min. ml of culture. (A600 unit) and 0.85 $ml.min.^{-1}$ $ml^{-1}$ of culture. (A600 unit) respectively. Thus 59.4A had a reduced rate of oxygen uptake of about 47% of that shown by the wild type.

This indicates that the spdl mutant was oxidizing acetate, although at a reduced rate. It also shows that it was able to respire but that its altered metabolism led to a reduced flow of electrons to oxygen and therefore presumably a reduced supply of ATP causing cells to become starved for energy. It is possible that the observed oxygen uptake was due to cells in the culture which had initiated sporulation. Sporulating cells do require some mitochondrial functions (Ephrussi & Hottinguer, 1951; Esposito et al., 1969), oxygen supply (Hansen, 1902 cited by Fowell, 1969), and an exogenous energy source for respiration (Pontefract & Miller, 1962; Miller, 1963a; Vezinhet et al., 1969) and the spdl mutants are able to use acetate as an energy source for sporulation (Vezhinet et al.,1979, see also Table 3.1). This all indicates that spdl mutants are able to derive enough ATP from the respiration of acetate to support sporulation, but not to maintain vegetative growth.

A previous hypothesis that the spdl mutants may have been to some extent uncoupled for ATP biosynthesis now seems untenable since an increased oxygen uptake would have been characteristic of this condition. Also, experiments with the uncoupling agent DCCD and the wild-type strain S41 did not show derepressed sporulation in uncoupled cells.

## Bacterial Cross-Feeding of spdl Mutants

On several YEPG plates with inocula of 59.4A, it was noticed that a contaminant bacterial colony had around it a halo of growing yeast cells, whereas elsewhere on the plate the yeast were unable to grow and sporulated extensively. The presence of the bacteria appeared to enable the mutant yeast to grow normally on this medium.

A pure culture of the cross-feeding organism was obtained, and was found to cross-feed 59.4A cells if streaked together with it on a YEPG plate, even if the cultures were not touching, indicating that some substance was diffusing through the agar. The cross-/

/feeding organism was found to be a Gram-positive coccus. The observation of cross-feeding was of interest as it probably indicated that the bacterium was converting glycerol to some other substance on which 59 4A cells were able to grow.

Growth on Preinoculated Media Liquid YEPG and YEPA media were preinoculated with the cross-feeding organism, cells were removed by centrifugation and the spent medium collected. This was then autoclaved and used as a substrate for S41 and 59.4A. Table 3.3 compares the abilities of S41 and 59.4A to grow on the pre-incubated media and on the original media. The spent YEPG medium acted as a good substrate for 59.4A whereas it was unable to grow

| | A600 reached after 24h incubation | | | |
|---|---|---|---|---|
| STRAIN | YEPG | YEPG* | YEPA | YEPA* |
| S41 | 4.5 | nd | 1.65 | 0 |
| S9-4A | 0.7 | 2.9 | 0.1 | 0 |

Table 3.3 Final A600 reached by S41 and 59.4A cultures on YEPG and YEPA liquid media, and on the same media after they had been preincubated with the cross-feeding bacterium, centrifuged and reautoclaved (YEPG* and YEPA*)

on the untreated YEPG. Neither strain was able to grow on spent YEPA. This indicates that the organism cross-fed 59.4A by converting glycerol into a substrate it could utilize. Examining the possible metabolic pathways involved, it was decided that the organism might be converting glycerol to dihydroxyacetone and excreting this, as this would yield energy for the bacterium, and other potential excretion products such as acetate and lactate are known not to support growth of spdl mutants.

Growth on Dihydroxyacetone The above possibility was examined by inoculating cells of strains S41 and 59.4A into media containing yeast extract, peptone and 10gl$^{-1}$ dihydroxyacetone. After 20h, the S41 culture had reached an A600 of 7.5 and there was no sporulation. The 59.4A culture had reached an A600 of 2.6 and had sporulated at a frequency of 10%. From Table 3.1, this indicated that dihydroxyacetone was intermediate in its properties as a growth substrate, between the fermentable substrates glucose and galactose, and the derepressing substrates glycerol acetate, lactate and pyruvate, since it supported some vegetative growth of the spd1-1 mutant, but not as much as the wild type, and the mutant sporulated on it but not as extensively as on other substrates. There was thus no hard boundary between derepressing and non-derepressing substrates. The fuller implications of this will be discussed later.

## Reversion* of spd1 Strains to Growth on Glycerol

One of the characteristic properties of spd1 mutants is the apparent ease with which they give rise to 'revertants'* able to grow on glycerol (Dawes, 1975). When the spd1 mutants are spread at high density on YEPG plates, small papillae (colonies of glycerol-growing yeast) appear in large numbers within a few days. The frequency of 'reversion' was estimated, as described in the methods section, by growing cells in YEPD, in which there was no selection pressure for glycerol growing mutants, and plating out onto GASP medium on which only 'revertant' colonies will grow. The cultures of 59.4A growing in YEPD were found to contain $(3.9 \pm 1.3) \times 10^{-4}$ glycerol growing mutants per cell.

## Discussion

The data presented in this chapter enable certain conclusions to be made about the nature of the defect in spd1 mutants. First, the derepressed sporulation characteristics of these mutants were/

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* As in the previous section, use of the words 'revertant' and 'reversion' here and elsewhere in this thesis, does not imply that the event concerned was a true reversion to wild-type at the spd1 locus.

/found to be associated, not with the specific substrate being utilized, but with the necessity of respiratory metabolism for growth. Secondly, the defect in ability to grow on glycerol was not due to inability to take the substrate up (Vezinhet et al., 1979) or due to any defect in the glycolytic pathway down to pyruvate, as the mutant was able to grow on glucose (Dawes, 1975) and dihydroxyacetone. This indicates that any metabolic defect in the spd1 mutant must be involved with the respiratory metabolism of substrates via the TCA cycle. The range of possible enzyme defects that might account for the phenotype of the spd1 mutation is further narrowed by the observation that it was able to utilize acetate as a substrate for sporulation (see Table 3.1). The glyoxylate cycle is essential for acetate utilization (Gosling & Duggan, 1971) and is used during sporulation (Tingle et al., 1973), most of the acetate taken up in the early stages being immediately converted to glutamate (J R Dickinson, pers. comm.). This means that glyoxylate cycle function is intact in spd1 mutants, and that their defect if in the TCA cycle, must be in a step not common to the glyoxylate cycle.

The behaviour of the mutant on dihydroxyacetone is interesting since the cells were neither fully repressed nor fully derepressed. If the dihydroxyacetone were being fermented via the second part of the glycolytic pathway, the net energy yield would only be one ATP molecule per dihydroxyacetone molecule, as one of the two ATP molecules generated would be required for prior phosphorylation of the dihydroxyacetone. Glycerol could not be fermented in this way as the reduced $NAD^+$ generated by the conversion of glycerol-3-phosphate, its immediate product, to dihydroxyacetone phosphate would be added to the NADH molecule generated in glycolysis, and only one of these could be regenerated to $NAD^+$ by the synthesis and excretion of lactic acid or ethanol, thus creating a situation in which the cell is unable to obtain $NAD^+$ in its oxidized form. Thus dihydroxyacetone, if fermented, should yield ATP, but only a single molecule per dihydroxyacetone molecule. The criterion for a derepressing substrate for spd1 mutants then, appears to be the balance of energy available from fermentation and respiration. Glucose yields a large amount of ATP from fermentation, and is not/

/derepressing, while acetate can yield no energy from fermentation, and is highly derepressing. Dihydroxyacetone does not yield energy from fermentation, but only a small amount compared with that available from respiration, and is a partially derepressing substrate.

One central problem in interpreting the data from spd mutants is that one cannot readily determine whether the initiation of sporulation or the cessation of growth is the primary event. The cells could be metabolically unable to utilize the substrate and therefore the ensuing state of starvation would induce sporulation, or the cell's metabolic imbalance could induce sporulation, preventing the cell from growing. The former suggestion is supported by the data indicating a close association of derepression with respiratory metabolism, while the latter suggestion is supported by the observation that cells are able to utilize the derepressing substrates as energy sources for sporulation. An attractive hypothesis combining the two is that spdl mutants are defective in an enzymic step in the TCA cycle not common to the glyoxylate cycle, but that the mutation confers reduced, but not abolished, activity of the enzyme. By this hypothesis, a small amount of ATP would be available from respiration, not enough to maintain vegetative growth, but enough to provide energy for sporulation.

The spdl mutants, therefore, show the salient characteristics of respiratory-deficient mutants. This is surprising since all the evidence in the literature (Ephrussi & Hottinguer, 1951; Vezinhet et al., 1969; Fast, 1973) indicates that respiratory sufficiency is important, if not essential, for sporulation, and that nearly all other respiratory-deficient mutants tested have severely reduced, or non existent, sporulation (see p.39). The spdl mutants, therefore, display the opposite to this normal situation; an apparent respiratory defect promotes sporulation. Therefore, studying the effects of carbon, nitrogen and energy metabolism on the sporulation of spdl strains should be instructive in establishing the nature of metabolic controls over sporulation. These aspects have been studied in more detail using continuous cultivation techniques, in a later chapter. However, before returning to the/

/subject, it was decided to concentrate on another very important point to emerge in this chapter.

The importance of the mutations restoring ability to grow on glycerol to spdl mutants can be seen by analogy with the lac (lactose utilization) operon of Escherischia coli. Superrepressed ($I^S$)E. coli mutants with mutations in the lacI gene (coding for the lac repressor protein) are unable to ferment lactose, but can give rise to lactose fermenting 'revertants' (using the terminology we have applied to the spdl situation) by a second mutation either in the lacI gene (to $lacI^-$) or in the operator gene (to $lacO^c$). Both of these latter mutations further identify the control functions in the lac operon (See Beckwith & Zipser, 1970 (eds.) "The Lactose Operon" Cold Spring Harbor Laboratory, New York, 1970). Thus the isolation of mutants from spdl parent strains with restored ability to grow on glycerol enables one to examine more closely the regulatory mechanisms in sporulation, especially if they prove to occur due to mutations at loci remote from the spdl locus. The suggestion that the spdl mutation confers a 'leaky' blockage in the TCA cycle could be due either to an enzyme structural gene defect, or a regulatory defect. The characteristics of any mutation which bypassed the block would be of considerable interest. Normal spontaneous mutational events occur at a frequency of 1 in $10^6$ to 1 in $10^7$ mutations per cistron per replication, with frequencies up to 1 in $10^4$ per replication at short mutational "hot-spots" within some genes (Auerbach, 1967; Drake, 1970). The observation that vegetative cultures of spdl strains without selective pressure for 'revertants' can nonetheless contain about 1 in $2.5 \times 10^3$ "revertants" shows that the rate at which such mutants arise must be several orders of magnitude above normal spontaneous mutation rates. The 'revertant' mutations are therefore highly interesting both in their own right as unusual genetic events, and also in the conclusions their existence allows to be made about the nature of the spdl mutation. These mutations have been studied more closely in the following chapter.

# CHAPTER 4 : THE PHYSIOLOGY AND GENETICS OF spd1 'REVERTANTS'

## Introduction

In Chapter 3 the very high 'reversion' rate of spd1 mutants to growth on glycerol was shown. It was not immediately obvious whether the 'reversion' events occurred at the spd1 locus or were due to a second-site mutation suppressing the effects of the spd1 mutation. A previous isolation (Calvert & Dawes, 1983) had shown that in one case at least this event was due to a mutation unlinked to spd1, and conferring asporogeny; this mutation was found in strain 69-10C which was used in experiments shown later. The second-site mutation to asporogeny is of great interest, if one considers the method by which it was isolated. Strain 69-10C was isolated due to its recovering the ability to grow on YEPG, in other words to grow when the parent spd1 strain (59.4A) hypersporulates. Since the mutation to asporogeny affects the 'decision' by the cell to grow or not, its effect on sporulation is probably due to a defect in 'switching' of the cell between cell division and sporulation, i.e. in initiation of sporulation rather than in any of the subsequent sequence of sporulation events. The work presented in this chapter used three protocols to try to isolate asporogenous initiation mutants. The first involved further isolation of diploid 'revertants' from a 59.4A culture, and relied on the 'revertants' failing to sporulate, and continuing vegetative growth due to inability to initiate sporulation. This method gives easy and rapid isolation of mutants, but subsequent genetic analysis is hindered since mutation to asporogeny makes difficult the sporulation and dissection of strains to establish epistasis, dominance and linkage relationships.

The second protocol was used to isolate asporogenous initiation mutants from continuous culture. The sporulation of yeast in continuous culture is treated in some detail in Chapter 6, which includes a more precise analysis of the selective advantage of asporogenous initiation mutants. In summary, in a population of/

/yeast cells growing in a chemostat with a small proportion of cells sporulating in each generation, any mutation that prevents the initiation of sporulation should confer a selective advantage since the population of cells carrying that mutation will not lose a proportion of growing cells to sporulation in each generation. Any other asporogenous mutation will not confer such an advantage. The method should enable the isolation of asporogenous initiation mutants from wild-type cells, and if successful would show that such mutations can arise in ways other than by 'suppression'* of spd1 mutants. The necessity of isolating such mutants from a homothallic diploid strain, however, generates the same difficulty in genetic analysis as the previous protocol.

The third protocol involved isolation of mutants from haploid spd1 strains. These were also isolated as 'revertants' to growth on YEPG. The rationale in this case is slightly different, since the second mutations do not prevent the initiation of sporulation but release the cells from arrest in the G1 phase of the cell cycle. It was of interest to determine whether these mutations also conferred asporogeny when in diploids in the homozygous state. This protocol has the advantage of ease of genetic analysis, since the mutations can be studied in both haploids and heterothallic diploids.

This chapter describes the isolation of mutants by these methods, and the characterization of the mutations obtained. Asporogenous initiation mutants will henceforth in this thesis be referred to as spo0. This usage is convenient, but does not conform to agreed nomenclature for mutations in yeast.

## Isolation of spo0 mutations

(i) Isolation from diploid spd1 strains Cells of strain 59.4A were inoculated onto YEPG agar at high density and 'revertant' strains were isolated. These were tested for their ability to sporulate, and/

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* The use of the term 'suppression' in this context does not necessarily indicate suppression of nonsense mutations by mutation of tRNA cistrons.

/those that sporulated sufficiently were dissected and the segregants characterized. The results obtained are shown in Table 4.1, together with the inferred genotype of the 'revertant' clones. It can be seen that three types of event were observed: revertants to wild type at the spd1 locus; heterozygous mutations to asporogeny; and homozygous mutations to asporogeny. It may seem surprising that no heterozygous revertants to wild type were obtained; presumably any such strains would have sporulated on reaching stationary phase, and produced homozygous daughter strains.

(ii) Isolation from diploid cells in continuous culture The precise method by which these mutants were isolated is described in Chapter 6. A single mutant strain, isolated from S41 in the carbon-limited chemostat experiment illustrated in Figure 6.1 was designated ch15 and used for further experiments described in this chapter. This strain failed to sporulate under any conditions tested.

| Number of Isolates | Percentage Sporulation | Dissection Results - Ratio of spore clones | | | Inferred Genotype | |
|---|---|---|---|---|---|---|
| | | Aspor-ogenous | De-repressed | Wild-type | spd1 Locus | spo0 Locus |
| 14 | 80 - 90 | 0 | 0 | 4 | SPD1/ SPD1* | SP0/ SP0* |
| 10 | 10 - 50 | 2 | 2 | 0 | spd1/ spd1 | spo/ SP0 |
| 10 | 0 | ND | ND | ND | spd1/ spd1 | spo/ spo |

Table 4.1 Analysis of revertants to growth on glycerol isolated from a 59.4A culture incubated at 30°C for two weeks on YEPG agar. Mutants were sporulated, where feasible, on KAc plates and dissected. Three phenotypes were distinguished from the growth on YEPG and sporulation on KAc. (ND) indicates dissection not feasible.

* SPD1 and SP0 refer to the dominant wild-type alleles at the spd1 and spo0 loci respectively.

(iii) Isolation from a haploid spd1 strain 'Revertants' to growth on glycerol were obtained from the haploid spd1 strain 168-2D, as previously described. The 'revertant' strains were backcrossed to the wild type strain 168-2A and where the reversion event had been due to a second mutation at a different locus from spd1, strains were isolated which carried the second-site mutation alone, without the spd1 mutation. These second-site mutations were then obtained in homozygous state in diploid strains, and the strains were tested for their ability to sporulate.

Using this protocol, the 20 'revertant' isolates in the original selection fell into four classes on subsequent analysis.

(i) Non-mating strains. Three were isolated which could not easily be analysed further.
(ii)Wild-type revertants. Three were found, in which the spd1-1 was not recoverable by backcrossing and the event was assured to have been a reversion to wild-type at the spd1 locus. They showed normal sporulation when mated to the wild type strain. It is also possible, of course that the event was a second-site mutation closely linked to spd1.
(iii)Unstable revertants. Six were isolated, which showed reduced sporulation when mated to the wild-type but which returned to the spd1 phenotype on further analysis.
(iv) Second-site mutations conferring asporogeny in homozygous state. Six were isolated.

The latter six, of class (iv), were assumed to have acquired one of the asporogenous initiation (spo0) mutations. Table 4.2 shows the dissection results obtained when these six original revertants were backcrossed to the wild type and dissected. As can be seen the spd1 phenotype was observed in segregant clones from all 'revertants' indicating recovery of the spd1 gene in separation from the second site 'revertant' mutation. It is difficult to make any conclusions from the ratios of the different types of ascus due to low spore viability which reduced the number of complete asci, and difficulties in scoring. The large number of tetratype asci compared with parental ditypes may indicate that some parental ditypes were scored/

| CROSS | SEGREGATION RATIOS GLYCEROL GROWTH : NON GROWTH | | |
|---|---|---|---|
| | 4 : 0 (PD) | 2 : 2 (NPD) | 3 : 1 (T) |
| rev 2 x wild type | 2 | 3 | 2 |
| rev 4 x wild type | 0 | 2 | 6 |
| rev 6 x wild type | 0 | 2 | 4 |
| rev 8 x wild type | 0 | 2 | 6 |
| rev 10 x wild type | 1 | 5 | 2 |
| rev 14 x wild type | 0 | 2 | 2 |

Table 4.2 Dissection results from back-crossing of haploid spd1 revertants to wild type, showing the number of asci in each dissection in which the glycerol non-growth characteristic had segregated as indicated. (PD) indicates parental ditype asci, (NPD) indicates non parental ditype, and (T) indicates tetratype.

/as tetratypes, possibly due to the presence of a petite mutation conferring the characteristic of non-growth on glycerol.

Next, it was important to establish whether these six spo0 mutations, together with the mutations in ch15 and 69-10C were all in the same gene, or whether mutations in a number of different genes can confer this phenotype.

## Complementation and Linkage Analysis of spo0 Mutations

Strains carrying mutations derived from the six haploid spo0 isolates, 69-10C and ch15 were crossed to each other for complementation analysis; complementation was assessed where the resulting cross sporulated to a signficant extent. The results are shown in Figure 4.1. The mutations fell into three complementation groups,/

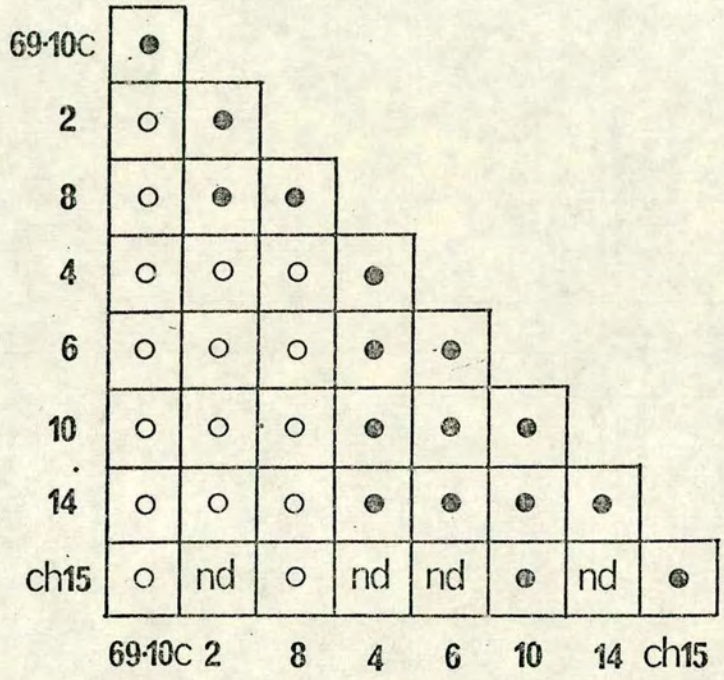



Figure 4.1 Complementation of mutations suppressing spd1 mutations as assessed by the ability of heterozygous diploids to sporulate on KAc medium. Complementation is indicated by (o); lack of complementation by (●). (nd) indicates that the cross was not made.

/one containing only the mutation in 69-10C, the second containing the mutations in rev 2 and rev 8 and the third containing the mutations from rev 4, rev 6, rev 10, rev 14 and ch 15.

A representative mutation from each complementation group was chosen in order to perform a linkage test to establish whether the three complementation groups reflected the presence of three separate mutations. The mutations chosen, those present in rev 8, rev 10 and 69-10C, were obtained in homozygous state in homothallic diploids, sporulated, and the few spores obtained were used for mating. In this way, when the mated strains were sporulated and dissected, the spores obtained would be homothallic and give rise to diploid strains, and so the presence of any asporogenous mutation could be detected directly. The results are shown in Table 4.3.

The three complementation groups clearly reflected the presence of mutations in three separate genes. The numbers of non-parental ditype and tetratype asci obtained indicated that there was no close linkage between these three mutations. These three separate mutations conferring the spo0 phenotype have been designated spo50, spo51 and spo 53.

| CROSS | TETRAD ANALYSIS | | | WILD-TYPE RECOMBINANT SPORES | |
|---|---|---|---|---|---|
| | PD | NPD | T | Fraction | Percent-age |
| spo50 x spo51 | 9 | 3 | 11 | 31/134 | 23 |
| spo50 x spo53 | 4 | 6 | 6 | 28/89 | 31 |
| spo51 x spo53 | 4 | 5 | 20 | 35/129 | 27 |

Table 4.3 Linkage analysis of spo0 mutations. Table shows tetrad analysis after dissection (PD indicates Parental Ditype axi, NPD, Non Parental Ditype, and T Tetratype), and percentage of spores showing wild-type sporulation. (designations of mutations is that subsequently given after identification of mutations; spo50 is the mutation from 69-10C, spo51 is the mutation from rev 8 and spo53 is the mutation from rev 10).

It can be seen from the data in Table 4.3 that although there was no evidence of close linkage, there was some evidence of centromere linkage. The segregation between spo51 and spo53 confirmed to a PD:NPD:T ratio of 1:1:4, indicating that there was no linkage at all between these two. The segregation between spo50 and spo53, however, was close to a 1:1:1 ratio of PD:NPD:T, indicating linkage of both mutations to their respective centromeres. Previous experiments on the segregation between spo50 and arg4-1, a centromere-linked marker have shown spo50 to be centromere linked (I.W.Dawes, pers. comm.) and so the evidence is strong that spo53 is situated close to a centromere. The ratios obtained in the segregation of spo50 and spo51 gave ambiguous indications, although since spo53 appears to be centromere-linked, and there was no sign of centromere linkage in the segregation between spo53 and spo51, it is probable that spo51 is not centromere-linked, and therefore that the PD:NPD:T ratio of 9:3:11 reflected a true ratio of 1:1:4, and that the initial figure of 9 for parental ditypes was in error. A possible cause of this is the appearance of petite strains. Some of the spo0 carrying strains showed poor viability and therefore any petite strains arising might not have been distinguished by their poor growth. Petite strains would have been scored as asporogenous, and therefore non-parental ditype and tetratype asci may have been scored as parental ditypes.

These experiments can only have established a minimum number of separate genes in the original isolations, as mutations which fail to complement could still be in separate genes. If they fail to complement however, the resultant cross cannot yield enough spores for dissection, and therefore linkage analysis is very difficult to perform.

## CHARACTERISTICS OF spo0 MUTATIONS

### Extent of Asporogeny

The three mutations spo50, spo51 and spo53 had similar effects/

/on sporulation. Under conditions in which wild-type populations sporulated to a level of 80-90%, a homozygous spo0 population sporulated to a maximum of 0.5% and normally less than 0.1%. When in heterozygous state, the mutations had a widely varying effect on sporulation and gave population sporulation rates between 30% and 60%.

There was, therefore, some gene dosage effect of these mutations on sporulation, but they were codominant It is interesting, however, that they were also dominant in their characteristic of restoring growth on glycerol to spd1 mutants (see discussion to this chapter).

Behaviour Under Starvation Conditions

One of the first observed characteristics of the spo0 mutations was that they conferred an apparent inability to adapt normally to starvation. When starved, strains carrying spo0 mutations lost viability very quickly. Figure 4.2 shows the loss of viability in four strains over a five-day period after reaching stationary phase in YEPA. The strains were S41 (wild type), 69-10C (spo50), 246-1B(spo51) and 247-20(spo53). The results clearly show a massive loss of viability in the spo0 strains compared with that in S41.

The spo0 strains also became grossly abnormal in shape when starved. Plates 4.1 and 4.2 illustrate the morphology of cells in a culture of 69-10C (spo50) under starvation conditions in YEPA. The cells became elongated and in many cases had apparently extended without undergoing full cytokinesis and separation. The cytoplasm of the cells appeared vacuolate and granular, probably indicating the approach of cell death. Plates 4.3 and 4.4 illustrate cells of S41 and 59.4A under the same conditions. The 59.4A cells sporulated extensively, as YEPA is a derepressing medium for them.

The formation of misshapen cells and pseudomycelial filaments is also characteristic of some cdc28 mutants incubated at the/

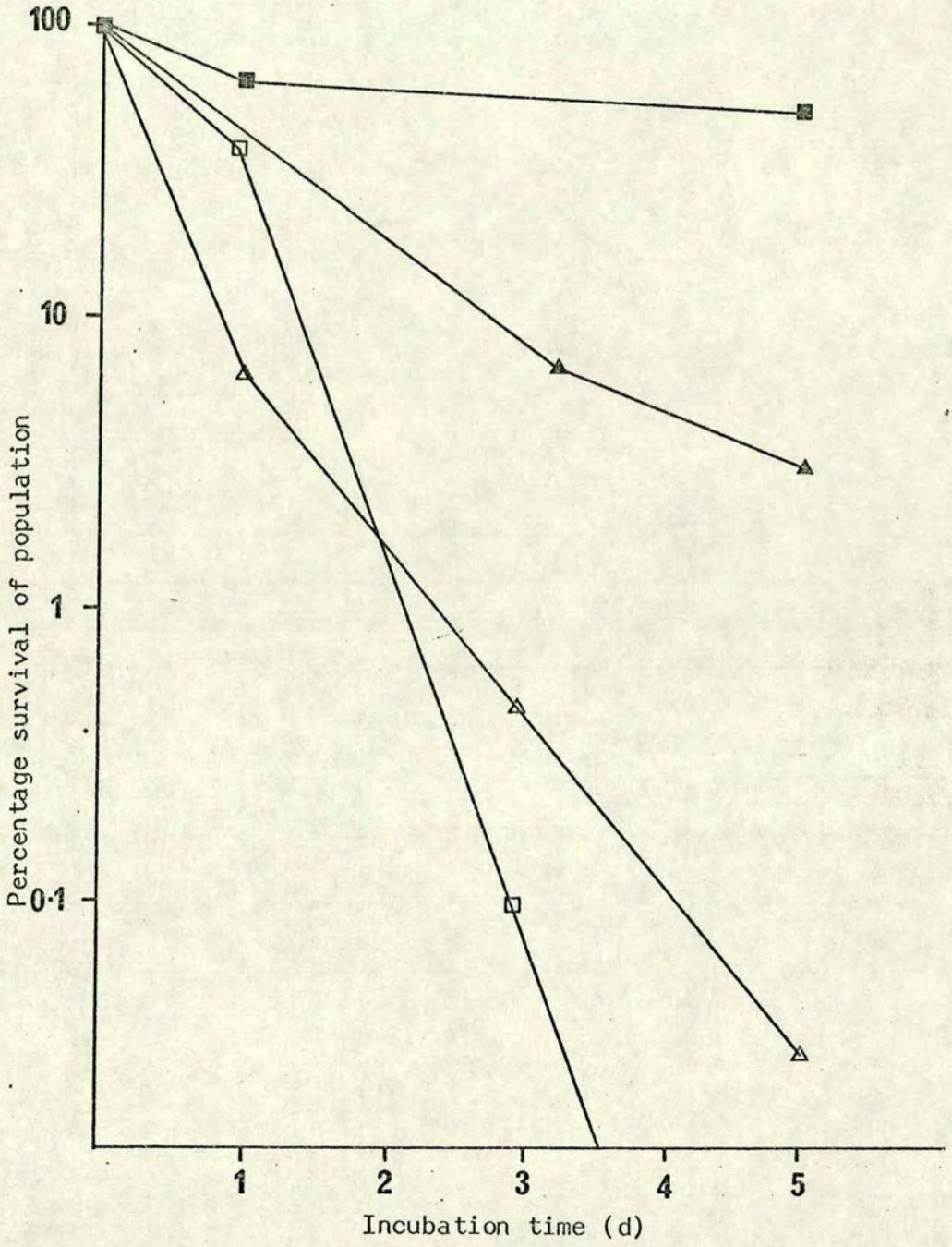



Figure 4.2 Loss of viability by spo0 strains under starvation conditions. Strains S41 (■), 247-2D (▲), 69-10C (△) and 246-1B (□) were grown to stationary phase in YEPA and incubated for 5 days at 30°C; viable counts were taken at various intervals.

Plates 4.1, 4.2, 4.3 and 4.4

Photomicrographs of strains under starvation conditions in YEPA. Plates 4.1 and 4.2 show strain 69-10C (*spo50* homothallic diploid). Plate 4.3 shows S41 (wild type diploid), and plate 4.4 shows 59.4A (*spd1* homothallic diploid). All photomicrographs were taken under phase-contrast illumination.

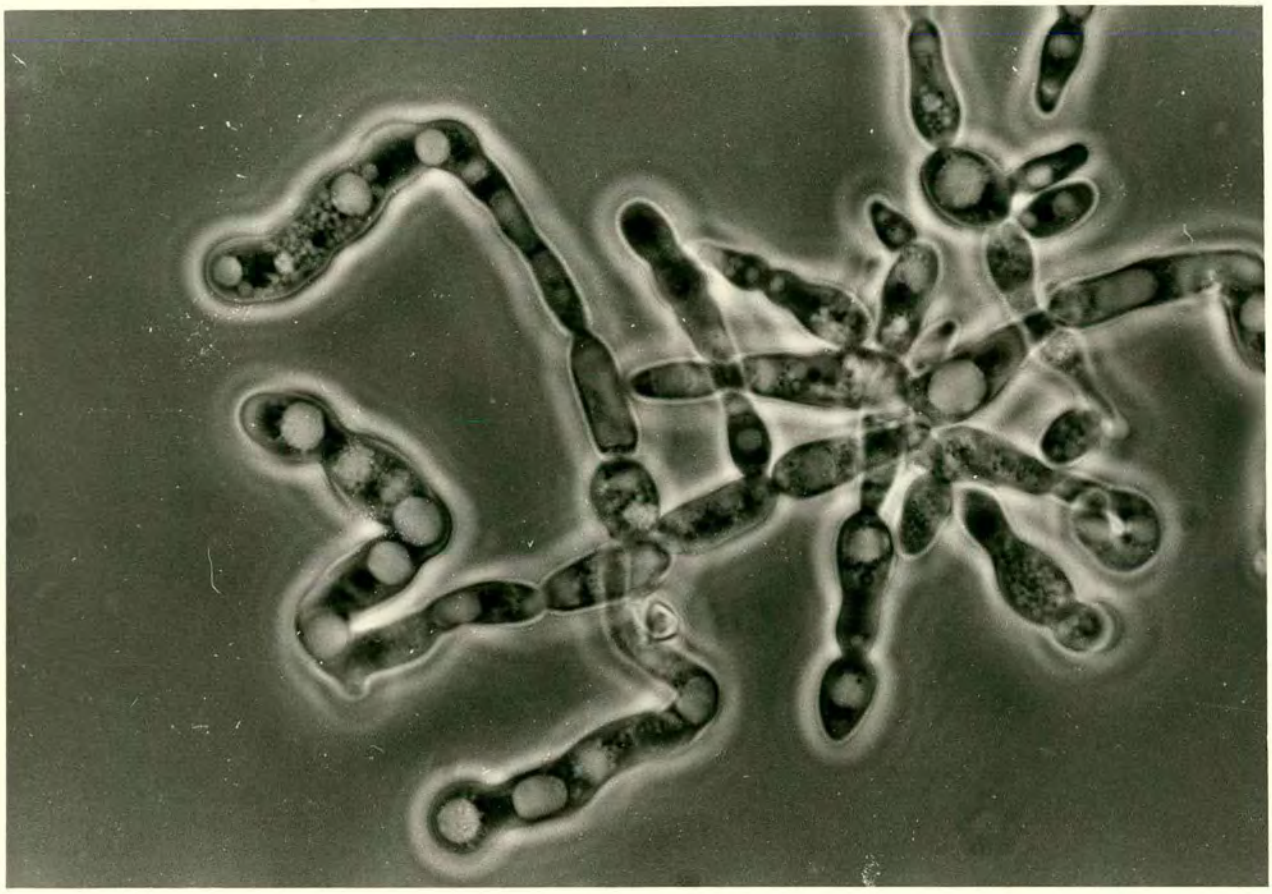
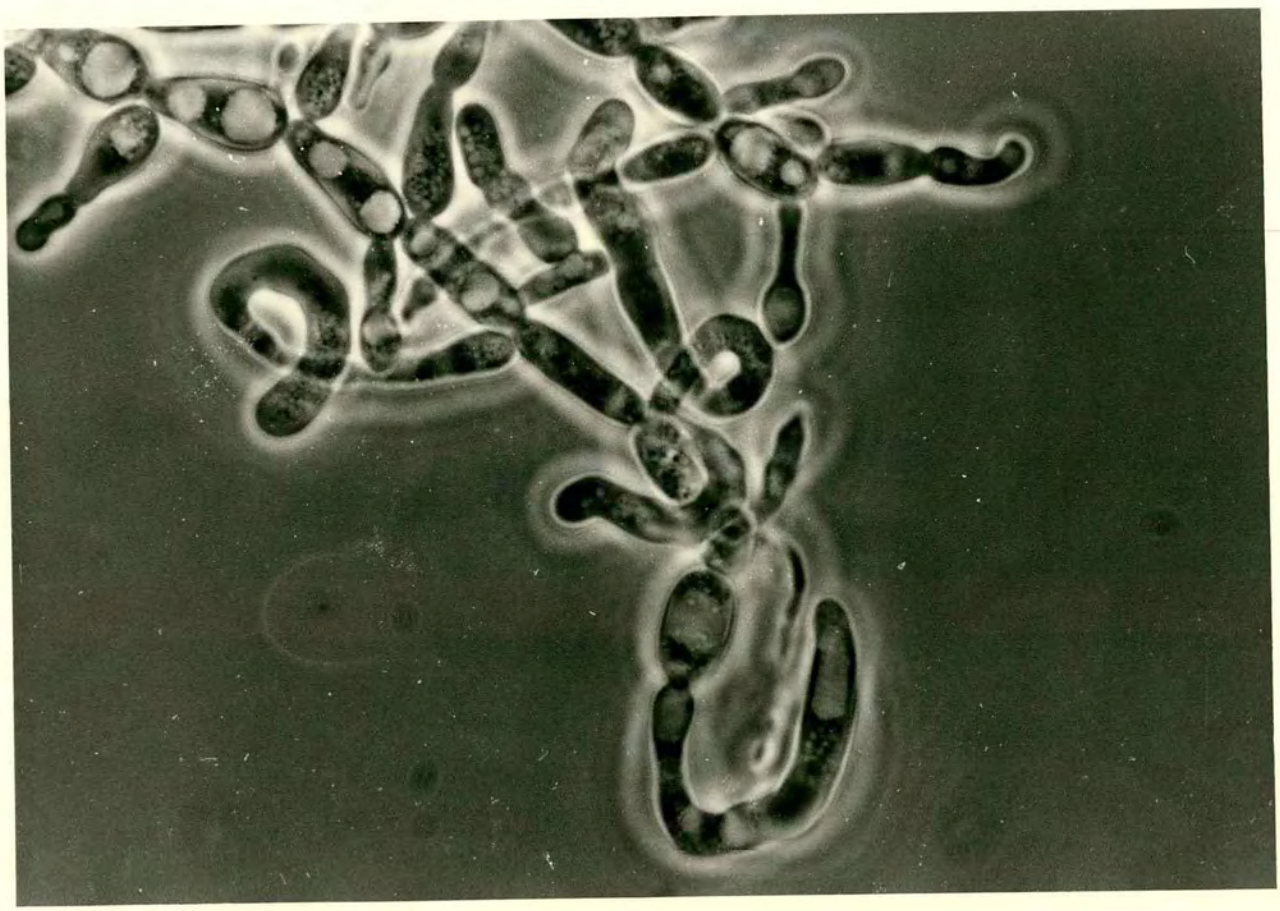


Plate 4.1

Plate 4.2



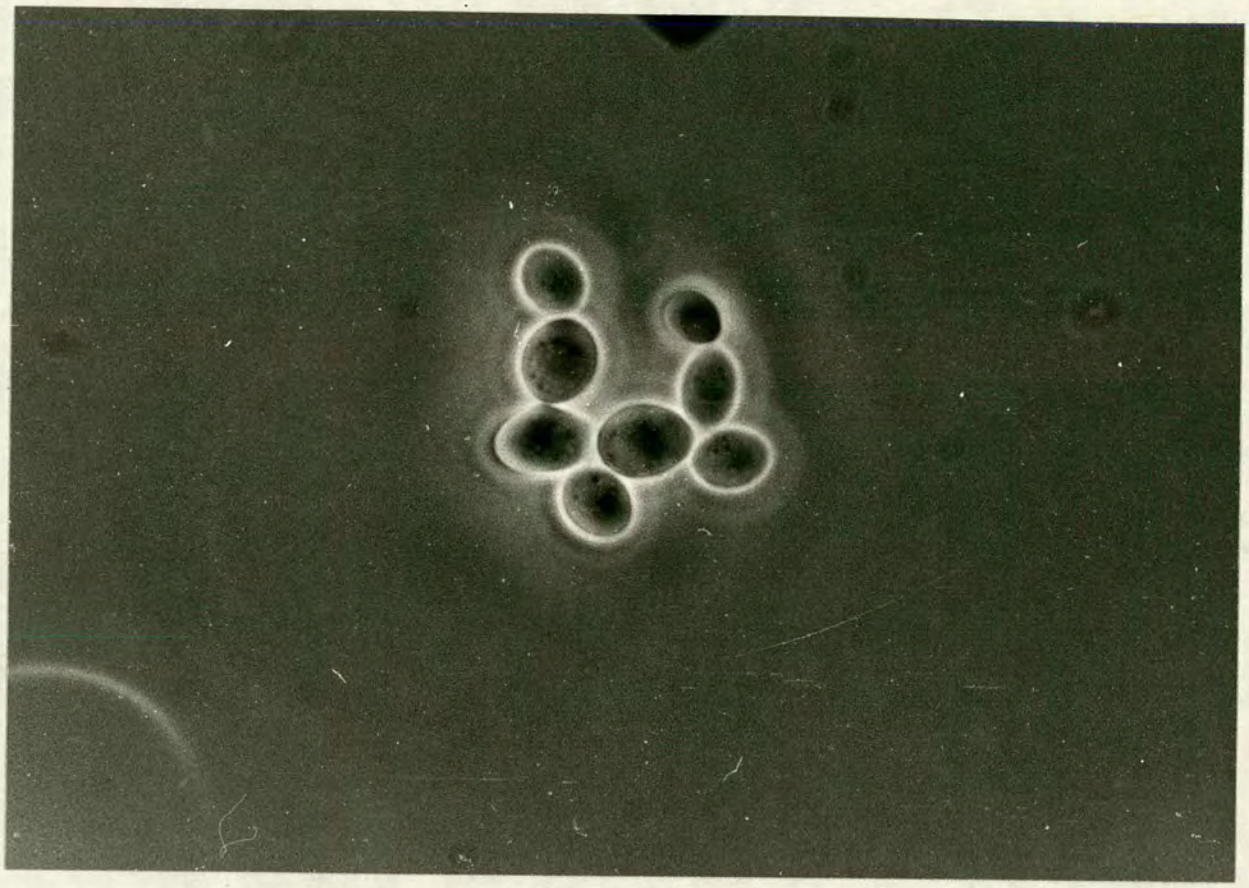


Plate 4.3

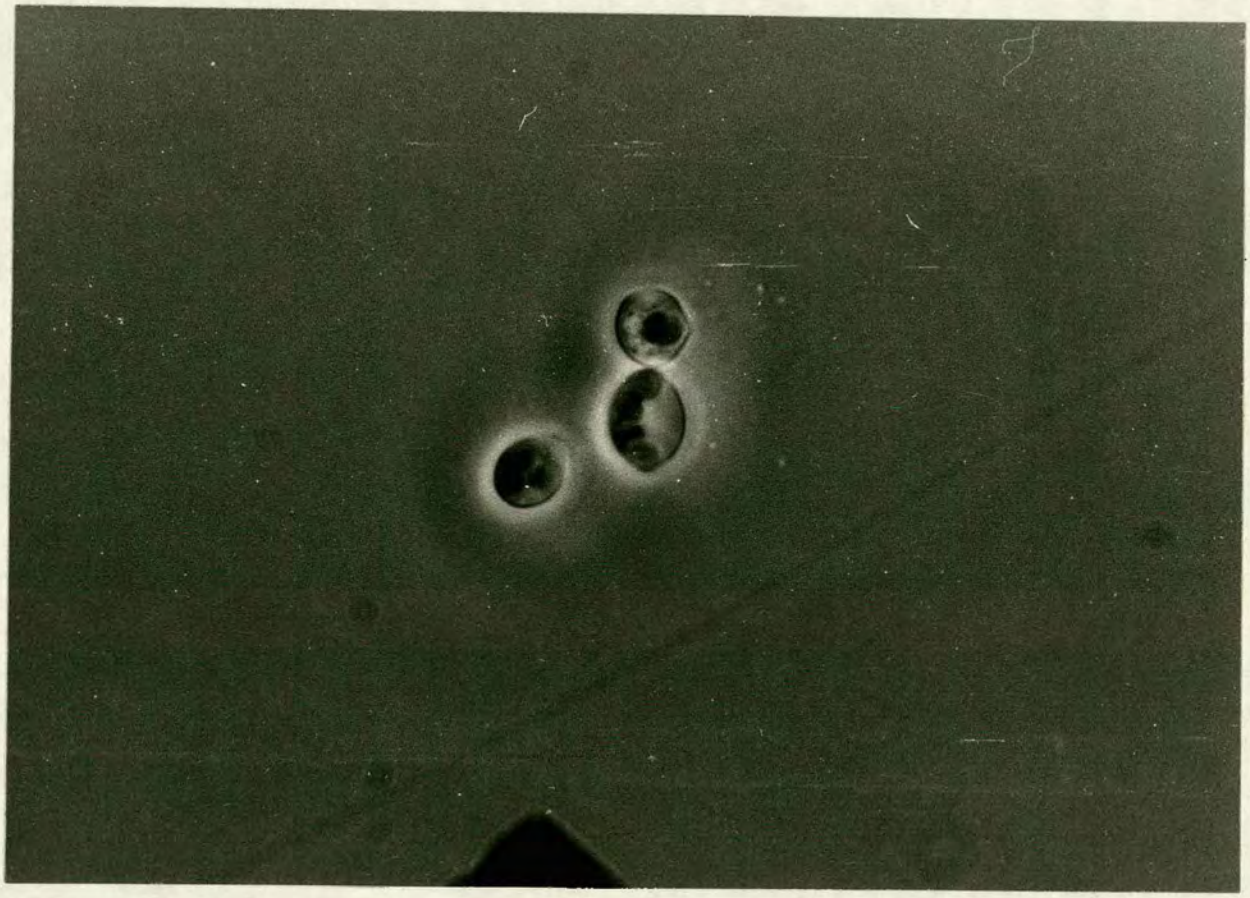


Plate 4.4

/restrictive temperature (Hartwell *et al.*,1973). The *cdc 28* mutation is, however, unlinked to *spo50*, *spo51* or *spo53*, as has been demonstrated later in this chapter.

Reduction of TTC

It has previously been suggested (Vezinhet *et al.*,1978; also pp.40 and70 ), that the *spd1* mutation may affect a central metabolic function involved in respiratory activity. Since *spo0* mutations apparently restore respiratory competence to *spd1* mutants as measured by growth on glycerol, an independent test of respiratory activity was required to establish that this was in fact the case. The test used was the reduction of TTC (triphenyl tetrazolium chloride). This colourless, soluble compound is reduced via a cell's respiratory chain to the insoluble red dye 2,3,5,triphenylformazan (Slater *et al.*, 1963). This substance was used by Ogur *et al.*(1957, 1965) and more recently by Boker-Schmitt *et al.*(1982) to detect colonies of respiratory-deficient yeast, by overlaying growing cultures with agar containing TTC. Respiratory-competent colonies stained red, while respiratory-deficient colonies remained white.

On YEPG, *spd1* colonies overlaid with TTC agar remained white, while both wild type and *spo0* colonies stained red, indicating respiratory competence in the latter. Surprisingly, however, on YEPD, on which both *spd1* mutant colonies and wild-type colonies remained white the *spo0* colonies stained red. The difference between wild-type and *spo0* strains in this respect is shown clearly in Plate 4.5. A heterozygous *spo53* strain, genotype $\frac{MAT_a HO\ arg4\text{-}1\ spo53}{MAT_\alpha HO\ arg4\text{-}1\ SPO53}$ was dissected and the resulting clones streaked onto a YEPD plate, grown, and overlaid with TTC agar. Plate 4.5 shows a clear 2:2 segregation of red and white clones; the red were homozygous *spo53* strains, and the white were wild-type strains. This indicated respiratory activity in the *spo53* strains even when growing on YEPD.

The reduction of TTC by cells growing on glucose is also characteristic of mutants in which mitochondrial biogenesis has been released from glucose repression (Boker-Schmitt *et al.*,1982) as determined by appropriate enzyme and cytochemical assays.

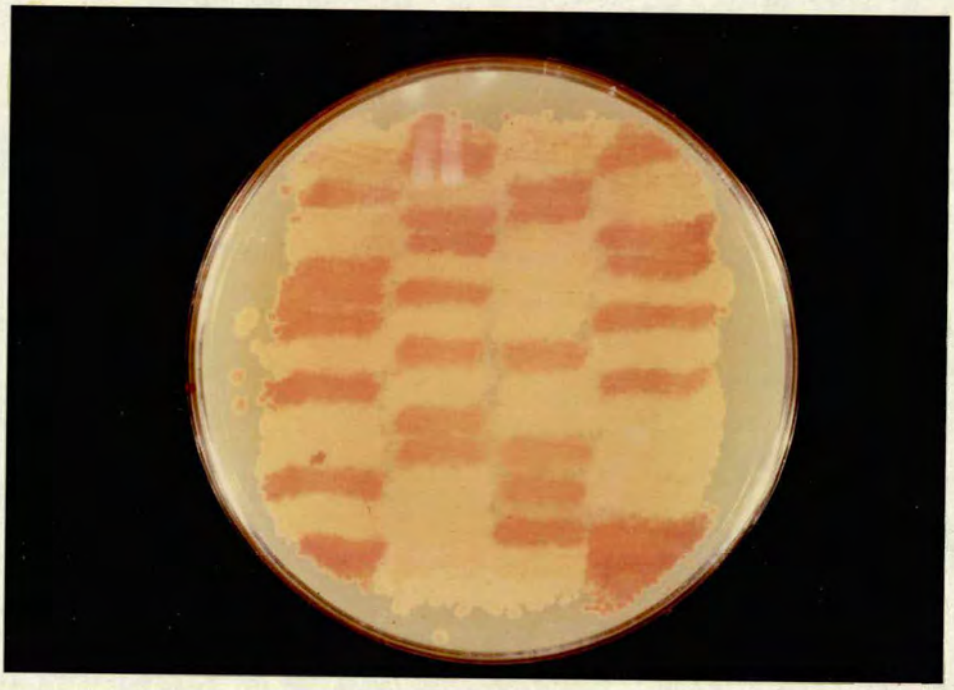


Plate 4.5 Reduction of TTC by spo0 clones compared with the wild type. A YEPD plate carrying the clones resulting from dissection of a heterozygous diploid spo53 strain was overlaid with TTC agar. The clones which stained red carried the spo53 mutation.

## Linkage Relationships between Mutations Involved in the Initiations of Sporulation

It has now been established that there are several mutations affecting the initiation of sporulation in yeast, including spd1, spd3, spo50, spo51, spo53, cdc25, cdc35, and possibly cdc28. Table 4.4 gives the dissection results of crosses made to establish whether any linkage existed between these mutations. The size-control mutations whi1 and whi2 were included since they also cause loss of viability on starvation as do the spo0 mutations, and also because they may be involved in cell cycle initiation (Carter & Sudbery, 1980; Sudbery et al.,1980). The linkage results are summarized in Figure 4.2. No close linkage was observed, but there was loose linkage of spo53 to spd1. In this cross the PD:NPD:T ratio was 14:1:15, indicating loose linkage. This enables an estimate to be made of the location of spo53 in the genome. The spd1 mutation defines a gene located 13.9cM from the centromere on the right arm of chromosome XV (I W Dawes, pers. comm.). This gives an appropriate map distance of 29cM between SPD1 and SPO53, and since SPO53 is centromere linked and therefore cannot be more than 30cM from the centromere, it must be distal to the centromere from SPD1 and, therefore, close to the map position of PET17. The random spore analysis of the cross between spo53 and spd1 was consistent with loose linkage: since if the value of 35% of spores as spd1 parental types was reflected in the spo53 parental types, there would be 70% parental type spores and 30% recombinant.

The result that whi1 and spo53 are not linked was obtained by inference, since there was no evidence from these data that whi1 was centromere-linked, and since spo53 was definitely centromere-linked, this indicated that there was no linkage between spo53 and whi1. This is supported by the fact that a similar argument held that there was no linkage between whi1 and spo50, and this was borne out experimentally.

The following crosses clearly showed no linkage: spd1 x spo50; spd1 x spo51; spd3 x spo50; spd3 x spo51; spd1 x whi1; spd1 x whi2; spo50 x whi2; spo53 x whi2; spo50 x whi1; spd1 x cdc 25; spd1 x cdc 35; spo50 x cdc 28; spo51 x cdc 28; spo53 x cdc 28; spo51 x cdc 25; spo53 x cdc 25; spo51 x cdc 35; spo53 x cdc 35.

| MUTATIONS USED IN CROSS (1) (2) | TETRAD ANALYSIS | | | RANDOM SPORE ANALYSIS | | | | |
|---|---|---|---|---|---|---|---|---|
| | PD | NPD | T | NUMBER OF SPORES | PARENTAL PERCENTAGE (1) | (2) | RECOMBINED PERCENTAGE WT | DOUBLE |
| spd1 x spo50 | 4 | 5 | 10 | 93 | 27 | | 73 | |
| spd1 x spo51 | 1 | 1 | 7 | 75 | 32 | | 68 | |
| spd1 x spo53 | 14 | 1 | 15 | 68 | 35 | | 68 | |
| spd3 x spo50 | 10 | 10 | 15 | 117 | 31 | | 69 | |
| spd3 x spo51 | 7 | 5 | 6 | 89 | 27 | | 73 | |
| spd3 x spo53 | 1 | 0 | 5 | 56 | 25 | | 75 | |
| spd1 x whi1 | 3 | 1 | 6 | 70 | 29 | 29 | 23 | 19 |
| spd1 x whi2 | 6 | 2 | 17 | 100 | 29 | 29 | 21 | 21 |
| spo50 x whi2 | 1 | 4 | 5 | 40 | 43 | | 57 | |
| spo51 x whi2 | 2 | 1 | 4 | 44 | 32 | 32 | 18 | 18 |
| spo53 x whi2 | 3 | 6 | 7 | 88 | 47 | | 26 | 27 |
| spo53 x whi1 | 2 | 4 | 5 | 36 | 11 | 11 | 39 | 39 |
| spo50 x whi1 | 2 | 1 | 5 | 32 | 25 | 28 | 25 | 22 |
| spd1 x cdc 25 | | | | 39 | 10 | 13 | 36 | 41 |
| spd1 x cdc 35 | | | | 35 | 11 | 23 | 26 | 40 |
| spo50 x cdc 28 | | | | 17 | 47 | | 53 | |
| spo51 x cdc 28 | | | | 29 | 48 | | 52 | |
| spo53 x cdc 28 | | | | 16 | 44 | | 56 | |
| spo51 x cdc 25 | | | | 16 | 38 | 30 | 13 | 19 |
| spo51 x cdc 35 | | | | 6 | 0 | 33 | 33 | 33 |
| spo53 x cdc 25 | | | | 11 | 27 | 18 | 49 | 9 |
| spo53 x cdc 35 | | | | 13 | 46 | 15 | 31 | 8 |

Table 4.4 Dissection results of crosses made to establish linkage between mutations involved in initiation of sporulation. Random spore analysis only is given for those crosses in which too few asci survived for tetrad analysis. Problems due to low spore viability were severe in crosses involving cdc mutations.

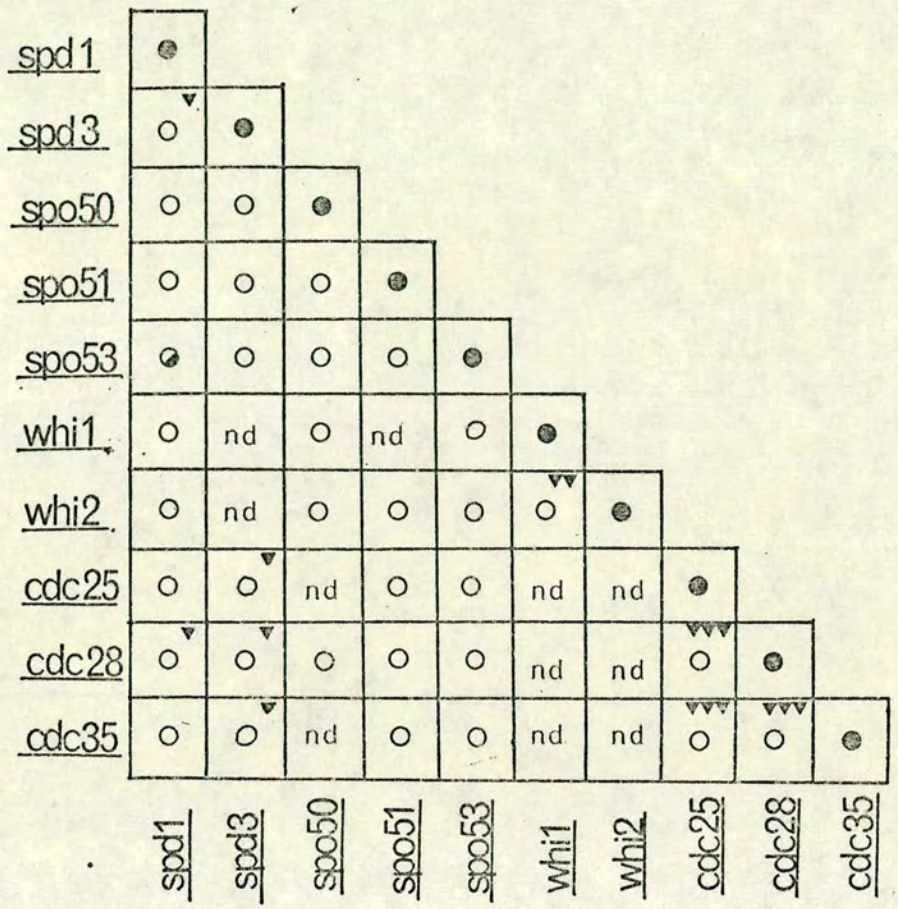



Figure 4.2 Linkage involving mutations involved in initiation. (●) indicates close linkage, (◐) indicates loose linkage, and (nd) indicates cross not made or spore survival too low for analysis. (▼) see Dawes & Calvert (in preparation); (▼▼) see Sudbery et al., (1980); (▼▼▼) see Hartwell, (1974).

Discussion

There are now probably seven mutations directly affecting the initiation of sporulation: spd1; spd3; cdc 25, cdc 35, spo50,spo51, and spo53 (cdc 28 is uncertain). Of these, spd1, spd3, cdc 25 and cdc 35 promoted the initiation of sporulation in cells carrying them, while spo50, spo51 and spo53 repressed it. The two spd mutations and the three spo0 mutations were all in genes intimately involved in the respiratory metabolism of the cell (Vezinhet et al., 1979). The spd1 and spo53 mutations were loosely linked, and were located close to the centromere on the left and right arms respectively of chromosome XV (Vezinhet et al., 1979;) . None of the other mutations appeared to be linked, although spo50 appeared to be centromere-linked.

The two spd and three spo0 mutations were unusual in their dominance and epistasis relationships. The spd mutations were mainly recessive, although they did show a slightly reduced ability to grow on glycerol and a slightly increased ability to sporulate when in heterozygous state (Dawes & Calvert, 1983). The spo0 mutations were mainly recessive in their effect on sporulation, although they did confer reduced sporulation in heterozygous state. They were, however, dominant in their effect of suppressing the phenotype of the spd1 mutation. The spd1 diploids that carried a heterozygous spo0 mutation showed no sign of reduced growth on glycerol. There was, therefore, some gene dosage effect in the influence of spd mutations on respiratory metabolism and sporulation, and in the influence of spo0 mutations on sporulation, but the epistasis between spd and spo0 mutations did not appear to depend on gene dosage. The signficance of these dominance and epistasis relationships has been discussed later in relation to the mode of suppression of the spd1 phenotype by spo0 mutations.

It was clear from the way in which the spo0 mutations were isolated, that spd1 spo0 double mutants were able to grow normally on glycerol. This indicates that an spd1 mutant has the potential to form the metabolic machinery to utilize glycerol, although it does not do so.

It/

It is now possible to extend the likely explanations for the defect in spdl mutants from those given in the previous chapter. The two major models suggested involved: (i) a defect in nutritional sensing; and (ii) a defect in an energy-yielding pathway.

In model (i), involving nutritional sensing, it is postulated that the cell is defective in its ability to assess its nutritional status, and although it is suspended in a medium in which it is able to grow, it responds as it would to starvation conditions. This could be conferred by a defect in a regulatory gene, or by a defect in the gene for an enzyme required for the process of nutritional sensing. Since very little is known about processes of nutritional sensing, this model is too vague to have any predictive value. It is also open to the objection that the spdl mutants do grow on glucose (Dawes, 1975) and therefore a different nutritional sensing system must be postulated for glucose. The mutants also grow on ethanol (Vezinhet et al., 1979) and this fact is difficult to accommodate in this model.

The second model postulates a defect in an energy yielding pathway, probably as indicated in the last chapter, in a function of the TCA cycle not common to theglyoxylate cycle. This could be a regulatory defect or a mutation in the structural gene for a particular enzyme in the cycle. Closely connected with this is the relative importance of the TCA andglyoxylate cycles in growth on glycerol and acetate. In growth on acetate which has a two-carbon skeleton, energy is available from direct respiration via the TCA cycle, but to obtain the three-carbon skeletons necessary for gluconeogenesis and synthesis of structural components, the cell used the glyoxylate cycle (Kornberg, 1966).

It is essential for glyoxylate cycle enzymes to be induced before yeast cells can grow on acetate (Gosling & Duggan, 1969, 1971). Glycerol, however, already has a three-carbon skeleton and might not, therefore, need glyoxylate cycle functions for its utilization. This again indicates, since the defect in spdl mutants is common to both acetate and glycerol utilization, that it is probably located in a TCA cycle step which is not necessary for glyoxylate cycle function.

Also, recent evidence (Dickinson et al., 1983) has shown that very soon after the initiation of sporulation on acetate, the acetate molecules are fed directly through the combined action of the TCA and glyoxylate cycles to glutamate. The glyoxylate cycle would then be necessary also to feed the glutamate through gluconeogenesis to form cell components. Since spd1 mutants were able to sporulate normally on acetate, their glyoxylate cycle functions appeared to be intact. Using this model, the defective, or defectively regulated, enzyme would be one of the following: isocitrate dehydrogenase; 2.oxoglutarate dehydrogenase, succinyl thiokinase, succinic dehydrogenase, or fumarase. Any such model must take into account the suppression of the spd phenotype by spo0 mutations. The reduction of TTC when growing on glucose is also characteristic of mutants released from glucose repression of mitochondrial biogenesis (Boker-Schmitt et al., 1982) as measured by enzymic and cytochemical assays, and it is therefore likely to be the case that spo0 mutants are derepressed in mitochondrial biogenesis or function, especially as they have had restored a mitochondrially-associated function. A model for the mode of suppression of the spd1 phenotype by spo0 mutations could, therefore, involve increased production or de-repressed function of mitochondria. If the spd1 mutation conferred, not an absolute block in an enzymic step, but a lowered efficiency, this would produce a lowered ATP yield from the TCA cycle. An increase in the availability of mitochondria would mean that, although each one was operating inefficiently, the total ATP yield might be large enough for growth. The enzyme affected in spd1 mutants may be 2.oxoglutarate dehydrogenase, which is the rate-limiting enzyme in the TCA cycle (Polakis & Bartley, 1965) and in which, therefore, a defect would have a severe effect on ATP yields. Also, blockage of this step would lead, via the reversible previous step mediated by isocitrate dehydrogenase, to a build up of isocitrate in the cell which could be removed via the glyoxylate cycle. If, as discussed earlier, glutamate production via glyoxylate cycle function is important in the very early stages of sporulation, this glutamate production may act to induce further sporulation functions in a dependent sequence. In the case of spd1 mutants, there may also be a build up of glutamate, or a previous intermediate, and this may induce sporulation functions.

It has been suggested later, in the chapter discussing size controls on sporulation, that there may be an unstable inhibitor of sporulation produced at a constant, gene-dosage determined rate during the cell cycle, which prevents sporulation until it has been diluted out by cell growth. If this were so, and if further production of the inhibitor were mitochondrially-mediated, an overproduction of mitochondria would effectively prevent sporulation.

It must be admitted that the above discussion is somewhat speculative. It does, however, provide testable predictions and if any part of it were correct, would provide valuable information about the control of sporulation. Possible future lines of enquiry will be taken up in the general discussion at the end of this thesis.

One of the general aims of this project was to isolate and characterize mutations in the initiation of sporulation in yeast. It can now be asked whether the spo0 mutations are in fact initiation mutations. The salient factor in determining whether a mutation is concerned with initiation is not its specificity to sporulation, but the point at which it affects the process. If it affects the probability of a particular pathway being followed at the branch point between cell divisions and sporulation, it can be said to be an initiation mutation, whereas if it affects the cell at a point subsequent to a particular 'decision' which has been made at the branch point, it is not an initiation mutation. The important question, however, is how mutations affect sporulation, not whether they conform to particular semantic definitions. Even if a mutation's primary effect were on the nutritional status or metabolic capacity of the cell, it could still be an initiation mutation, as the initiation of sporulation is presumably regulated by concrete physiological factors and not conceptual 'decisions'. Since nutritional status is a 'trigger' for sporulation, any mutation influencing it may influence the initiation of sporulation.

From their method of isolation, it is clear that spo0 mutations cause cells to continue growth in conditions under which/

/wild-type cells sporulate. This shows that these mutants are prevented from sporulating early in the process, before they become committed to it. It does not, however, prove that they are initiation mutants, but is consistent with such a classification. They show none of the early morphological changes associated with sporulation, such as changes in refractility and rounding-up, both of which occur in the very early stages of sporulation. Ajam (1981) has shown that cells of strain 69-10C (spo50 diploid) undergo no premeiotic DNA synthesis under sporulation conditions, and also that they display a few early, but no late, sporulation-specific polypeptide changes that can be detected in normal sporulation by polyacrylamide gel electrophoresis (Wright & Dawes, 1979; Ajam et al., 1981). This indicates that they may perform some very early sporulation function before returning to vegetative growth. The spo0 mutations, therefore, appear to be involved either at, or soon after, the point of initiation.

The spo0 mutations also prevent the cell from taking up a normal stationary phase, as shown by their loss of viability and aberrant morphology. This lends support to the suggestion of Pinon (1978) that stationary phase is a distinct morphological phase, termed Go, and not simply arrest in G1. This has been discussed previously (see p.32). This conclusion is made since the spo0 mutants are able to grow vegetatively and are therefore able to undergo G1 processes, and so their defect is in Go and not G1. If G1 were not a distinct phase, this would be impossible.

The spo0 mutants, however, do not show normal morphology when growing under nutritional stress; some cells apparently fail to undergo proper cytokinesis and cell separation, while still increasing in size. It is possible, if the cells are proceeding part way into sporulation before returning to vegetative growth, that in doing so they bypass some function involved in cytokinesis. This would only hold for cells which had initiated sporulation which would explain why the aberrant morphology only appeared under nutritional stress, and then not in all of the cells.

In this chapter, the characteristics conferred by the three/

/classes of asporogenous mutations that were isolated have been discussed, together with those of the derepressed sporulation mutations. However, to make sensible statements about the role played by individual genes in initiation, it is necessary to determine how these interact with each other. In the next chapter the interaction between the spo50, 51 and 53 mutations and the spd1 and spd3 mutations, and also that between spd1 and whi1 and whi2 will be considered.

# CHAPTER 5 : INTERACTIONS BETWEEN MUTATIONS INVOLVED IN INITIATION

## Introduction

It has been shown that at least seven genes are directly or indirectly involved in the initiation of sporulation, and in the previous chapter it was shown that these, identified by mutations, were separate and unlinked, except for loose linkage between spo53 and spd1. Since control mechanisms involve several gene products (For a review of bacterial regulatory systems, see Travers, 1971) it is important to determine whether there is any interaction between initiation mutations, and especially relationships of synergy or epistasis. In this chapter is examined the interaction between three groups of mutations; the spo0 asporogenous initiation mutations, and the whi size control mutations (Carter & Sudbery, 1980; Sudbery et al., 1980).

By virtue of their method of isolation, the spo0 mutations suppress spd1, but it is not evident that they also suppress spd3 or how this suppression operates. The possibility that spo0 mutations suppress spd mutations via suppression of nonsense mutations by altered tRNA codons has been examined.

The relationship between spd mutations and whi mutations is interesting because they have opposite effects under starvation conditions; the spd mutations arrest the cells (Vezinhet et al., 1979) while the whi2 mutation causes continued division (Carter & Sudbery, 1980; Sudbery et al., 1980).

## Suppression of spd mutations by spo0 mutations

Homothallic diploid strains heterozygous for one of the spo0 mutations and one of spd1 or spd3 were built, sporulated and dissected. Table 5.1 shows the resulting segregation of glycerol growth and non-growth characteristics in the spore clones. If there were no suppression of the spd mutations by the spo0 mutations, all the/

| MUTATIONS INVOLVED IN CROSS | RATIO OF SPORE CLONES IN ASCI, GLYCEROL GROWERS : NON GROWERS | | |
|---|---|---|---|
| | 2 : 2 (PD) | 4 : 0 (NPD) | 3 : 1 (T) |
| | Number of asci | Number of asci | Number of asci |
| spo50 x spd1 | 4 | 5 | 10 |
| spo50 x spd3 | 10 | 10 | 15 |
| spo51 x spd1 | 1 | 1 | 7 |
| spo51 x spd3 | 7 | 5 | 5 |
| spo53 x spd1 | 14 | 1 | 15 |
| spo53 x spd3 | 1 | 5 | 0 |

Table 5.1 Epistasis of spo0 mutations. Dissection products from strains heterozygous for one of the spd mutations and one of the spo0 mutations.

/resulting asci would show 2:2 segregation of glycerol growth:non growth. As Table 5.1 illustrates, however, there were significant numbers of asci which segregated 3:1 and 4:0 in all of the dissections, showing that all three spo0 mutations suppressed both spd1 and spd3. All three spo0 mutations, spo50, spo51 and spo53 are therefore epistatic to both spd1 and spd3.

## Nonsense Suppression of the spd1 Mutation

It was thought possible that the spo0 mutations were suppressing the phenotype of the spd1 mutations via suppression of nonsense alleles by mutations in tRNA codons. To test this, a strain was built carrying the spd1-1 mutation and leu2-1, an amber-suppressible nonsense allele (Hawthorne, 1976). This strain, designated 206-1B (genotype $\frac{\text{MAT}\alpha \quad \text{HO} \quad \text{leu2-1} \quad \text{spd1-1}}{\text{MAT}\alpha \quad \text{HO} \quad \text{leu2-1} \quad \text{spd1-1}}$) was inoculated onto YEPG plates and strains 'revertant' for growth on glycerol were isolated as previously described. These clones were then tested for their growth on leucine deficient plates. Of 48 independent clones 'revertant' for growth on glycerol, none had reverted to growth on leucine deficient plates. The spd1-1 mutation, therefore, does not co-revert with leu2-1. In further experiments (Calvert & Dawes, 1984), the spd1-1 mutation was found not to corevert with any of a series of ochre, amber and opal suppressible alleles, and was also found not to be suppressible by several nonsense-suppressor mutations.

This evidence indicates that spd1-1 was not a nonsense-suppressible mutation, and also that the spo0 mutations were not themselves suppressors of nonsense mutations.

## Interactions between spd and whi mutations

For the following tests, five homothallic diploid strains were constructed which were (i) homozygous for whi1; (ii) homozygous for both whi1 and spd1; (iii) homozygous for whi2; (iv) homozygous for both whi2 and spd1; (v) homozygous for spd1 only. Figures 5.1 and 5.2 show the behaviour of these strains under vegetative growth and starvation conditions, as measured by the distribution of cell/

/lengths in the culture. Cell length was used as an estimate of the cell size, since microscopic examination was essential to measure the size of budded cells, asci, and cells in clumps, and also since a rapid reliable method was needed to measure large numbers of cells. The limitations of measuring cell size by this method were appreciated.

The results in Figures 5.1 and 5.2 show that the presence of spd1 did not cause whi1 or whi2 cells to be significantly larger, nor did it prevent the reduction in size of the cells when they reached stationary phase. Its presence, however, does enhance the ability of both whi1 and whi2 strains to sporulate on resuspension in the KAc medium. The sporulation frequencies of the double mutants, however, were intermediate between those of the spd1 and whi strains, indicating that the whi mutations inhibit sporulation, while the spd1 mutation enhances it.

From the dissection of the crosses made between whi1 and whi2 carrying strains, and spd1 and spd3 carrying strains used to obtain data for Table 4.4, it was observed that neither of the whi mutations suppressed the inability of spd1 or spd3 strains to grow on YEPG, although they severely affected sporulation, as in the above experiment.

From these results, it appears that whi1 and whi2 do not affect the inability of spd1 and spd3 strains to grow on glycerol, spd1 does not affect the size-control defect in whi1 and whi2 strains, and that the cumulative effect of these mutations is additive, rather than interactive.

In these experiments, illustrated in Figures 5.1 and 5.2, it was also noted that only the large cells in the cultures were sporulating. On examining this more quantitatively it was found that there were no spores in cells shorter (in their longer axis) than 4.5μm. Further, on examining the distribution of cell sizes in the vegetative cultures from which these asci came, it was/

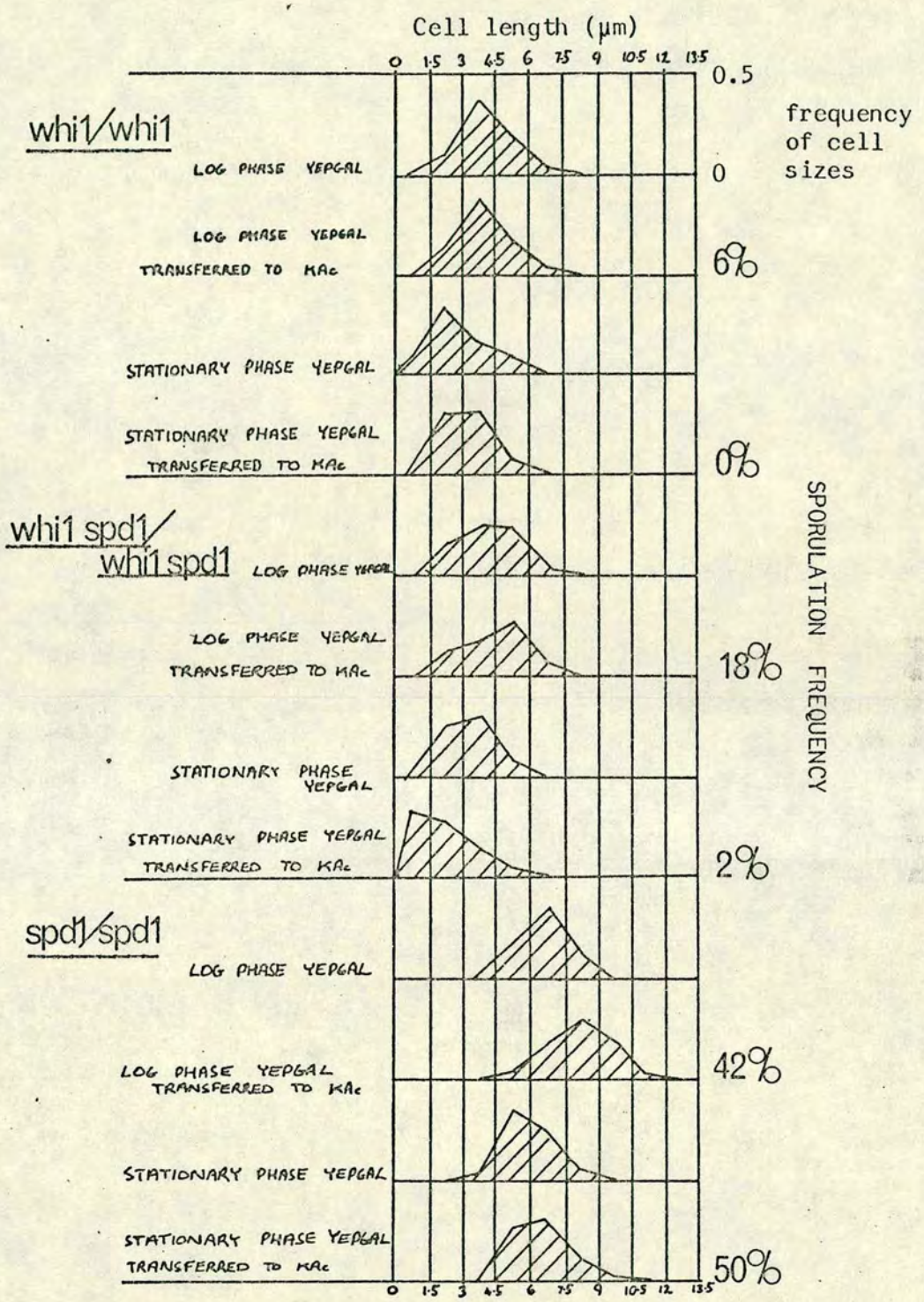



Figure 5.1 Behaviour of whi1, spd1 and whi1/spd1 strains under vegetative growth and starvation conditions, as measured by cell length distribution. (See text for details). At least 100 cells were measured for each condition.

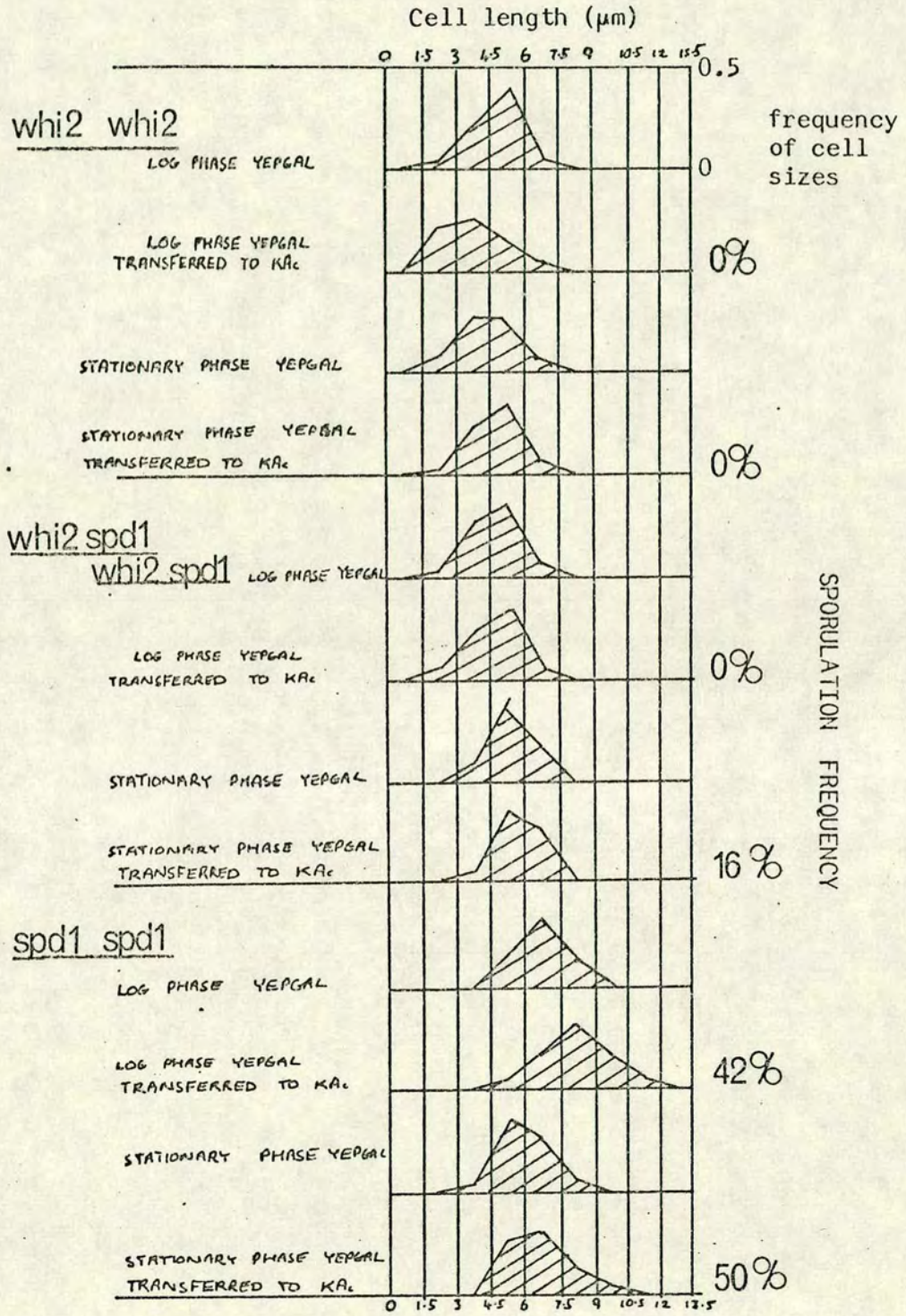



Figure 5.2 Behaviour of whi2, spd1 and whi2/spd1 strains under vegetative growth and starvation conditions, as measured by cell length distributions. (At least 100 cells were measured for each condition).

/observed that the level of sporulation in the KAc culture was correlated with the proportion of cells in the presporulation parent culture which were longer than 4.5 μm. These preliminary data can be interpreted to indicate that a cell-size control exists over sporulation such that only cells over a certain size can sporulate. This suggestion has been investigated further in the next chapter.

## Discussion

In the initial section of this chapter it was shown that all three spo0 mutation, spo50, spo51 and spo53 were able to suppress both spd1 and spd3 in their effects of preventing growth and causing sporulation on glycerol media. This has certain implications for the mode of this suppression; clearly suppression by spo0 mutations is not specific to just spd1 mutations and therefore the products of the wild-type SPO0 genes do not solely affect the expression of the SPD1 gene. It also indicates a fairly close relationship between the SPD1 and SPD3 gene functions. This is further support for the hypothesis postulated in chapters 3 and 4, that the spd1 mutation causes a deficiency in the respiratory metabolism in the cell, which is compensated for by a large increase in the mitochondrial content of the cell. If this were the case, spo0 mutations should suppress any 'leaky' mitochondrial respiratory-deficient mutation, and not just spd mutations. Other explanations for the mechanism of the suppression are, of course, possible.

It is now possible to suggest what wild-type functions may be mutant in spo0 strains. The products of these genes appear to be involved in the repression of mitochondrial biogenesis by glucose. Their importance for sporulation is presumably due to the intimate connection of mitochondrial activity with the ability to sporulate. The effect of spo0 mutations on sporulation may not, therefore, be a direct one, but act through their influence on the mitochondria. Alternatively, the primary effect may be on the initiation process, and the secondary one on the mitochondria. In either case, the interdependence of respiration and sporulation is again illustrated.

The results of the studies of the whi1 and whi2 mutations and their effect on sporulation indicate that there may be a cell-size control over the initiation of sporulation. This would explain the data on the interactions of whi1, whi2 and spd1. On its own, a whi mutation reduces average cell size to such an extent that few cells are large enough to sporulate. Although the additional presence of the spd1 mutation does not increase the average cell size to allow a high rate of sporulation, it enhances the sporulation capability of those cells large enough to sporulate. It is also likely to arrest starved cells before they undergo enough divisions to decrease dramatically in size under the influence of whi2. In a double mutant, therefore, carrying both whi and spd1, a sporulation frequency intermediate between that of spd1 and whi mutants alone might be expected.

The data presented in this chapter can only be preliminary in establishing the existence of a size-control over the initiation of sporulation, since they involve cells already mutant in size control over cell division, they involve cells of different genotypes in different media, and measure cell length, which is not an entirely adequate parameter in estimating cell size.

The existence of a cell size control over the initiation of sporulation has been examined further in the following chapter.

# CHAPTER 6

# CONTINUOUS CULTIVATION STUDIES OF CELL SIZE AND NUTRITIONAL CONTROL OVER INITIATION OF SPORULATION

## INTRODUCTION

The two distinct elements of control over initiation of sporulation, the well established nutritional control and the cell size control suggested in Chapter 5 can both be studied to advantage by using continuous cultivation techniques. In the case of nutritional regulation, continuous culture provides a means of controlling, and maintaining constant the nutritional stress on cells limited for a particular nutrient, and so the events involved in the initiation of sporulation can be made to occur continuously in the chemostat rather than being confined to a short stage of rapidly changing conditions in a batch culture. Indeed, the possibility of obtaining a continuous culture that continuously sporulates provides information about the control of sporulation (See discussion on p.123 ). Secondly, by changing the dilution rate in a chemostat it is possible to examine the response of cells over a variety of levels of nutritional stress while maintaining them on the same basic medium and limiting nutrient.

In the case of cell-size control, continuous culture allows one to obtain cultures containing cells of a variety of sizes, and the proportion of large and small cells in the culture can be varied since it is dependent upon the dilution rate (Lorincz & Carter, 1979; Johnston *et al.*, 1979). This then provides a method for obtaining cells of varying sizes for sporulation studies without recourse to the use of size control mutants.

### Nutritional Control

Preliminary experiments on the initiation of sporulation in carbon- and nitrogen-limited chemostats showed glucose to be unsuitable as a carbon-source as even low levels of it repressed sporulation. Since *spd1* mutants did not grow on non-fermentable substrates it was necessary to use a fermentable substrate to obtain comparable results with mutant and wild-type strains, and/

/galactose, which is fermentable but does not repress sporulation, was used for all further experiments. Also, complex media containing peptone or yeast extract were found to repress sporulation and therefore semi-complex media containing defined carbon and nitrogen sources were used, together with Yeast Nitrogen Base which supplied supplementary nutrients. Under these conditions, a readily-assimilable nitrogen source was found to be essential for the support of growth in chemostats, and ammonium sulphate was used in all further experiments. The above criteria were used to design the galactose- and ammonia-limited media used in the nutrient control experiments described in this chapter.

(i) Galactose-limited chemostats

Wild type strain Cells of the wild-type strain S41 were inoculated into a galactose-limited chemostat medium as described in the materials and methods section. The dilution rate was set at $0.0227h^{-1}$ for the duration of the run.

Figure 6.1 shows the behaviour of the culture during the 500h after inoculation, in terms of its cell density (estimated by turbidity at 600nm), the percentage of asci and the percentage of asporogenous, tetrazolium-reducing mutants in the culture.

After inoculation, the turbidity increased exponentially as the culture grew up. The turbidity reached a peak level when the culture became limited, followed by a sharp decrease. At this point, therefore, a large proportion of cells had ceased growing and the culture was starting to wash out. The subsequent appearance of asci in the culture shows that some, at least, of those cells that had ceased growing had become initiated to sporulation; it should be noted that there is a delay between the initiation of sporulation and the appearance of mature asci. In the following period the percentage of asci increased to a peak value which remained steady for a short period of time, during/

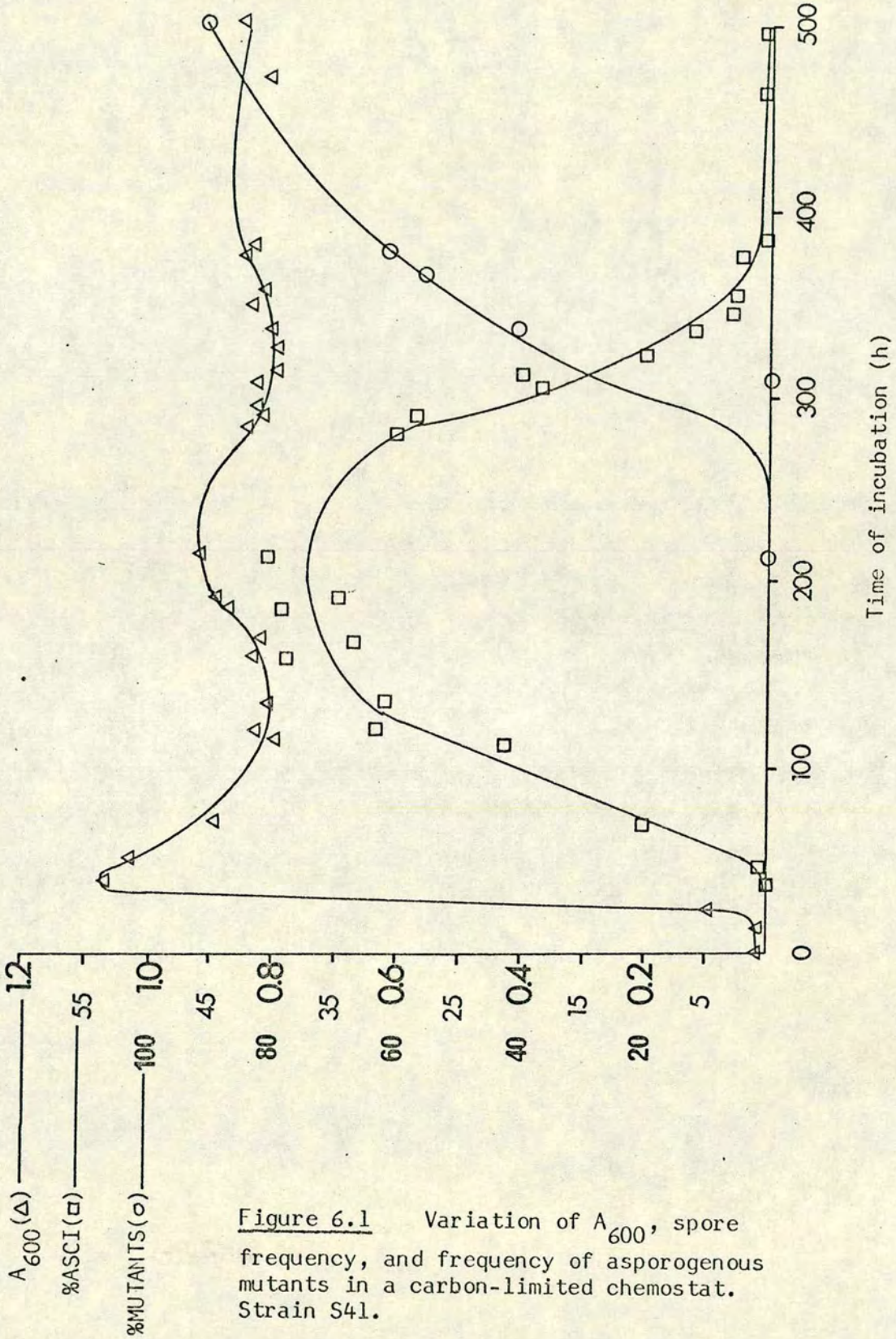



Figure 6.1 Variation of $A_{600}$, spore frequency, and frequency of asporogenous mutants in a carbon-limited chemostat. Strain S41.

/which the fall in cell density stopped and then began to rise again as the cells prematurely adapted to a higher growth rate to allow for the continuous loss of a proportion of growing cells through sporulation. The system is therefore able to adjust itself to an equilibrium condition where the continuous loss of cells through sporulation is balanced by an increased growth rate. It is suggested that the explanation is as follows. The treatment used follows that of Dawes & Thornley (1970) for Bacillus Subtilis , and is applicable to yeast sporulation in continuous culture provided that spore germination does not contribute significantly to population growth.

The theory predicts that a steady-state culture can be obtained in which a certain proportion of cells are initiated to sporulation in each generation. The vegetative cells in the population ($Nml^{-1}$) contribute to population growth by virtue of a specific growth rate ($\nu\ hr^{-1}$). In a chemostat, the rate of population growth is modified by the dilution rate ($Dhr^{-1}$) and by the initiation of cells to sporulation at a specific rate ($Khr^{-1}$) such that, to a first approximation,

$$\frac{dN}{dt} = (\nu - K - D)N$$

(Dawes & Thornley, 1970; Herbert, Elsworth & Telling, 1956)

Both $\nu$ and K are functions of the limiting substrate concentration (S) (Herbert, Elsworth & Telling, 1956). Monod (1942) has shown empirically (for mass growth rate, which is usually analgous to number growth rate) that

$$\nu = \nu_m \frac{S}{Ks + S}$$

where $\nu_m$ = maximum specific growth rate of the organism at saturating levels of the limiting nutrient

and Ks = saturation constant

The /

The variation of K with s has only been determined empirically for Bacillus subtilis, in which the K was found to be a linear function of $\nu$.

$$K = a - b\nu \qquad \text{(Dawes \& Thornley, 1970)}$$

Solution of the chemostat equations for the effect of limiting substrate concentrations cannot be adequately performed as information is not available about the extent of substrate utilized by sporulating cells, nor about the homogeneity of sporulating cells of various ages in utilizing substrates.

It is possible to use the above relations to explain the nature of the results shown in Figure 6.1.

During the initial phase, when the culture was growing at a maximum rate, all of the cells were vegetative and

$$\nu = \nu_{max}$$
$$\text{and } K = 0$$

The cells divided at $\nu_{max}$ until just before limitation was reached, at which point S would have begun to fall, together with $\nu$ which is dependent upon S. For a non-sporulating organism $\nu$ would have fallen to the value set by the dilution rate D.

However, as the culture became limited, sporulation would have begun to be initiated. If there was a time lag between the onset of nutrient starvation and the initiation of sporulation, the vegetative cell population would overshoot the ultimate steady state cell concentration to approach that achieved by a non-sporulating organism. Following the peak value of N, there was heavy initiation of sporulation, as demonstrated by the large numbers of asci appearing from 20h after the peak.

At this point however the cells were growing at a specific growth rate $\nu$ approaching D, and since/

$$\frac{dN}{dt} = (\nu - K - D)N,$$

$\frac{dN}{dt}$ became negative and the culture started to wash out. As the culture washed out, however, the rate of consumption of the limiting nutrient would have decreased, leading to an increase in (s) and therefore would have risen again until a new equilibrium was established at which $\nu - K = D$.

This explanation would predict that the equilibrium value of $\nu$ and N were reached by oscillation on either side of the equilibrium values, and there is some indication of this in the data presented in Figure 6.1.

If we now consider the appearance of the tetrazolium-reducing asporogenous mutants which rapidly took over the culture after about 300h incubation, it is clear that they had a considerable selective advantage.

In fact, any asporogenous mutant arising would have grown at a rate $\frac{dN}{dt}$ where

$$\frac{dN}{dt} \text{ (mutant)} = (\nu - D)\, N$$

since it would not have lost any vegetative cells through sporulation.

Thus: $$\frac{dN}{dt} \text{ (wild type)} = (\nu - K - D)\, N$$

and $$\frac{dN}{dt} \text{ (mutant)} = (\nu - D)\, N$$

Assuming that the mutant adjusted its growth rate according to the limiting nutrient concentration in the same way as the wild type, $\nu$ would have the same value in both the above formulae. Since D is also the same in both formulae, $\frac{dN}{dt}$ (mutant) would have been larger than $\frac{dN}{dt}$ (wild type) due to the element K. Since the proportion of *asci* reached 35%, K would have been a large element/

/compared with $\nu$ and D and therefore the mutant increased in numbers very quickly at the expense of the wild type and rapidly displaced it completely.

Thus the very large increase in tetrazolium-reducing mutants which occurred after about 300h operation can be explained by the above treatment, assuming that these mutants were asporogenous initiation mutants (See Chapter 4 for a discussion of this).

Derepressed Sporulation Mutant in Galactose-Limited Continuous Culture

Figure 6.2 shows the behaviour of 59-4A, an spd1 diploid, grown under the same galactose-limited chemostat growth conditions as used for the wild-type strain S41 in the previous section. In the case of 59-4A the same general behaviour was observed, but the stages were more pronounced, and changes occurred more rapidly. The turbidity rose in the same way initially but fell much more dramatically after reaching the peak corresponding to the onset of limitation. The percentage of asci also rose much more rapidly, reaching a peak after 100h instead of 200h in the wild type. In this case, after reaching a maximum, the percentage of asci fell again, presumably because the initial drop in turbidity after the peak value was enough to allow a large rise in limiting nutrient concentration and therefore a rise in growth rate and drop in sporulation rate. The mutants arose much sooner, after about 100h, whereas they arose only after nearly 300h in the wild type culture. It is also notable that the initial peak turbidity was not as high as in the wild type culture, presumably because the cells had already started to sporulate before the maximum turbidity was reached. This is confirmed by the early appearance of asci less than 20h after the peak of turbidity had been reached, showing that significant initiation had taken place before the culture reached maximum turbidity. Thus the spd1 mutant showed substantially the same behaviour as the wild type in the carbon-limited chemostat, but its reactions to carbon limitation and relief of it were much greater, faster but less sustained than was the case with the wild-type and the conditions in the chemostat therefore fluctuated much more. The implications of this will be examined later.

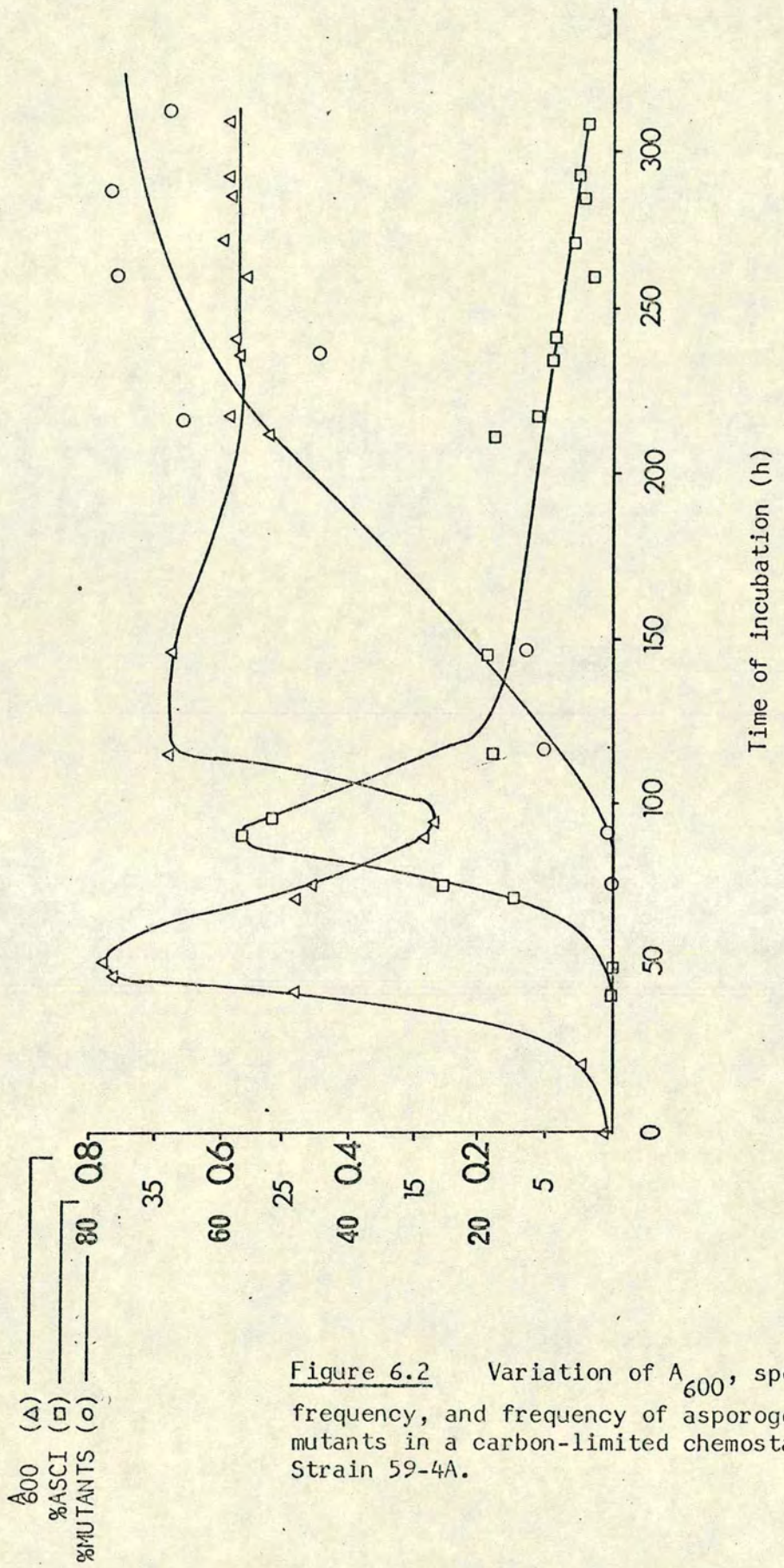



Figure 6.2 Variation of $A_{600}$, spore frequency, and frequency of asporogenous mutants in a carbon-limited chemostat. Strain 59-4A.

(ii) Sporulation in Nitrogen-limited chemostats

The above results indicate that it is possible to establish and study sporulation of yeast in a continuous culture, although care must be taken that the appearance of asporogenous mutants does not affect the results. They also indicate that sporulation is not necessarily on all or none response by a population to the limitation of nutrients, but that at any given state of nutrient limitation there is a particular probability of an individual cell initiating sporulation before it can divide, and that this probability will manifest itself in a certain proportion of the cells in a culture initiating sporulation.

To extend these findings further, it was necessary to follow the effects of other types of limitation on sporulation, and specifically on the extent of initiation of sporulation at different dilution rates. Since cells that have been initiated to sporulation cease to grow, once a particular cell has initiated sporulation there is a particular probability, dependent upon the dilution rate, that it will be washed out during the time between initiation and the point at which it is detectable as a mature ascus. Thus the proportion of mature asci in a steady state continuous culture will be an underestimate of the rate of initiation of sporulation. The factor by which it is an underestimate will be dependent upon the dilution rate and the time elapsed between initiation and maturation of asci, and the latter quantity must therefore be known, in order to calculate initiation rates. A preliminary experiment was therefore performed using N-limitation (conditions described in Materials and Methods) and a sudden shift in dilution rate to determine, if possible, the time taken from initiation to formation of mature asci. If the rate of initiation of sporulation (K) is a function of the dilution rate, then by suddenly shifting down the dilution rate when a culture is in steady state, mature asci should increase suddenly at a time Th. after the shift down, and Th. will therefore be a measure of the time from initiation to maturation of asci.

A culture of cells of strain 59-4A was inoculated into a nitrogen-limited chemostat as described in the materials and methods section. The culture was allowed to reach steady state at a dilution rate of $0.0895h^{-1}$ and the percentage of *asci* in the culture was monitored. The dilution rate was then reduced to $0.0239h^{-1}$ and the percentage of *asci* in the culture was monitored over the following 50h. Figure 6.3 shows the results. Between 12h and 22h after the change in dilution rate, a sudden increase in the proportion of *asci* was observed, the midpoint of this "burst" occurring 17h after the change in dilution rate. It was assumed that the *asci* thus appearing were initiated to sporulation at the time of change of dilution rate, and thus the average time from initiation to maturation of spores in the chemostat was 17h. These results also show that the rate of initiation of sporulation is therefore a function of dilution rate, and therefore of the growth rate for limiting substrate concentration.

Sporulation Under Steady-State Conditions It was found that in the nitrogen-limited medium described in the materials and methods section, both S41 cells and 59-4A could sporulate in steady-state in a chemostat, the conditions in the chemostat remaining constant while a small proportion of cells in the culture sporulated in each generation. In contrast to carbon-limited chemostat cultures which were extremely unstable, the nitrogen-limited chemostat cultures could remain in a stable steady-state for at least 500h and up to 1000h. Figure 6.4 illustrates the results from culture of S41 cells and 59-4A cells at a range of dilution rates, showing the extent of budding and the extent of initiation of sporulation. The percentage of budded cells was measured by microscopic examination of a sample from the chemostat. The percentage of cells being initiated to sporulation was calculated from the percentage of mature *asci* as determined by microscopic examination using the formula

$$\theta_i = \theta_{sp} \times e^{D\tau} \qquad \text{(Dawes \& Mandelstam, 1970)}$$

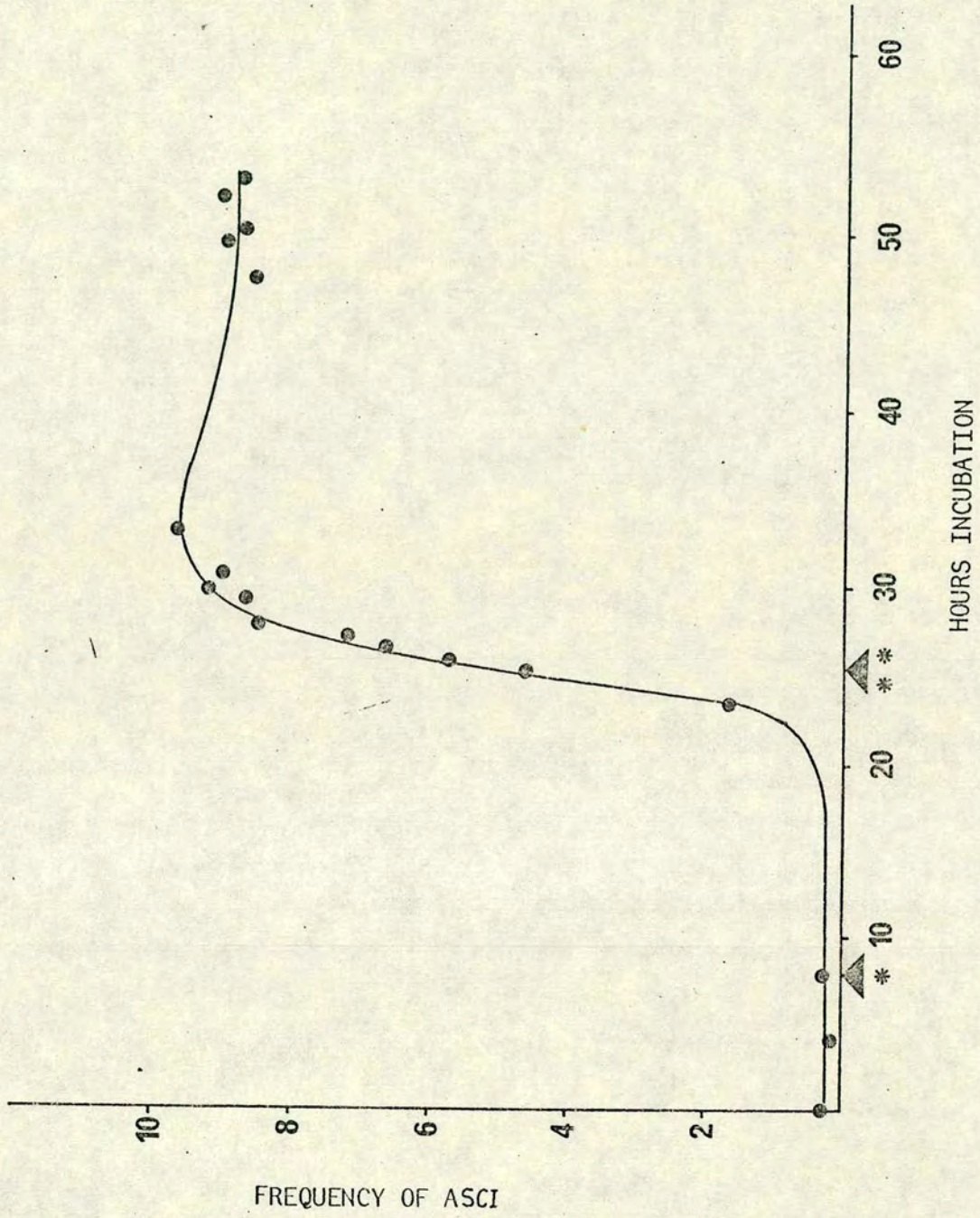



Figure 6.3 Time course of sporulation after switch-down of dilution rate in a nitrogen-limited chemostat. (*) indicates time of switch-down, (**) indicates mid-point of rise in spore frequency.

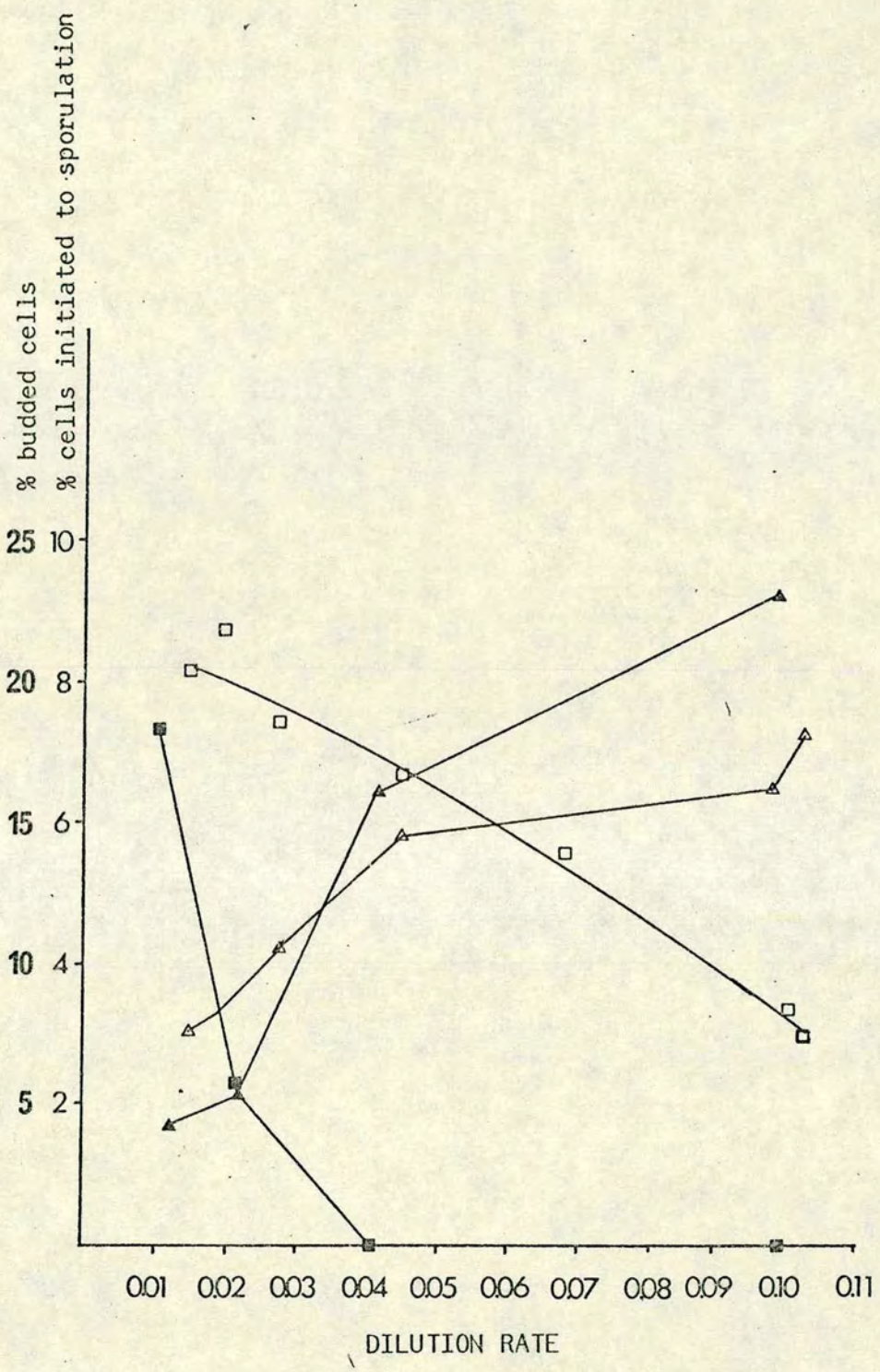



Figure 6.4 Graph showing percentage of cells initiated to sporulation for strains S41 (■) and 59-4A (□) and percentage of budded cells for S41(▲) and 59-4A (△) over a range of dilution rates in a nitrogen-limited chemostat.

where $\theta i$ is the percentage of initiated cells, $\theta sp$ is the percentage of *asci*, D is the dilution rate and $T$ is the time from initiation to maturation of *asci*. The use of this formula accounts for the wash-out of cells between initiation of sporulation and maturation of *asci*.

As is clear from Figure 6.4, S41 and 59-4A cultures both showed a graded response to different dilution rates. At low dilution rates, and therefore low growth rates, the extent of budding was low and the proportion of initiated cells (and therefore the initiation rate) was high, where as at high growth rates the proportion of budded cells was high and the initiation rate low. The extents of budding in S41 and 59-4A cultures were similar to each other over the range of dilution rates, but the extent of initiation of sporulation varied widely. 59-4A cultures showed a gradual decrease in initiation with increased dilution rate, whereas S41 cultures showed a dramatic decrease, ceasing to sporulate at all at dilution rates above $0.04h^{-1}$. The implications of this for the control of sporulation will be examined later.

## Cell Size Control over Sporulation

The following experiments were performed to test the suggestion made in Chapter 5 that there is a cell size control over sporulation.

Dependence of Sporulation on Cell Length For these experiments, two strains, S41 and 59-4A were used, differing in their genotypes only by the possession by 59-4A of the spd1 mutation. Three different chemostat media were used, to obtain the desired range of cell sizes, as described in the legend to Figure 6.5. In each case, a culture was allowed to reach steady state in the chemostat and a small sample taken. The distribution of cell lengths was measured, as described in the Materials and Methods section, by microscopic examination, and then a larger sample was taken, centrifuged and the pellet was washed twice in KAc medium and resuspended in the KAc medium containing penicillin to reduce possible contamination. The culture was incubated for 48h at $30^{o}C$/

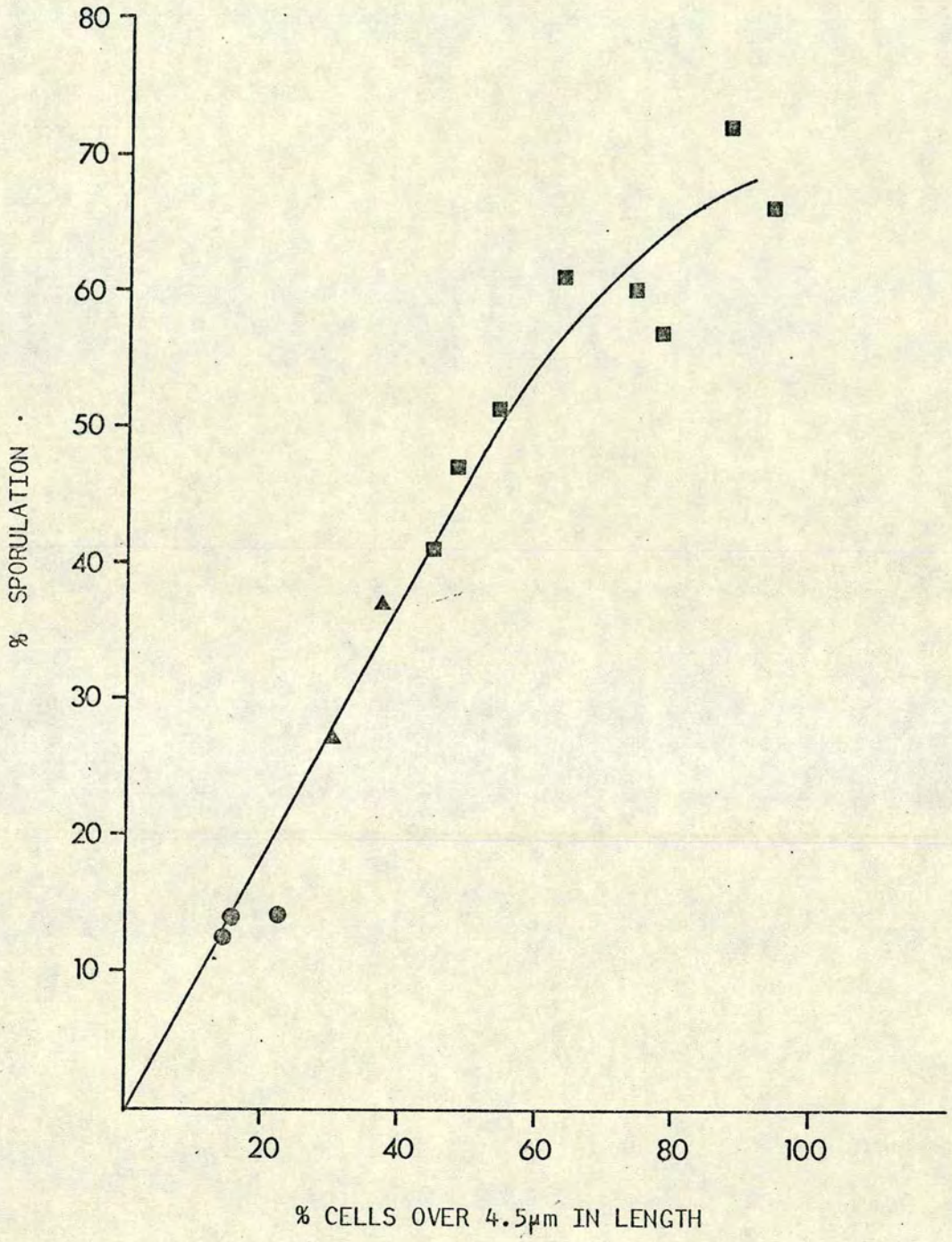



Figure 6.5 Correlation of the percentage sporulation of a culture transferred to KAc, and the percentage of cells in the inoculum larger than 4.5mm in length. (●) 59-4A grown on chemostat medium A, (▲) S41 grown on chemostat medium B, (■) S41 grown on chemostat medium C.

/after which the percentage of asci was measured (full details of the procedure are given in the Materials and Methods section). Figure 6.5 shows the relationship between the final percentage of asci in the cultures and the percentage of cells in the chemostat culture which, immediately prior to initiation, were greater in length than the 4.5μm critical size indicated in Chapter 5. At low to intermediate sporulation rates, the percentage of asci was directly proportional to the percentage of cells larger than the critical size, and almost equal to it. Presumably the reason that the values were not precisely equal is that not all of the cells that were large enough to sporulate in fact did so. The highest frequency of asci obtained under these conditions was 80%, and this explains the fact that the graph departs from proportionality and levels out as the percentage of asci approaches this value. Although the data given in Figure 6.5 are less satisfactory on several counts, since different media and different strains were used, and cell length is not necessarily the best measure of cell size, the results do clearly show a dependance of sporulation on cell size, and indicate that there may be a critical cell size below which cells do not sporulate. It was therefore decided to test this dependence further.

Dependence on Cell Volume In this case, the experimental procedure was exactly the same as that outlined above, except that a single strain and a single chemostat medium were used, and the distribution of cell volumes was measured instead of cell length. This was done by microscopic examination under x100 magnification using an eyepiece graticule and measuring the major (a) and minor (b) diameters of each cell. Assuming the cells to be prolate spheroids, the formula

$$V = ab^2/6$$

where V is the cell volume, can be used to calculate the cell volume (Wheals, 1982). At least 100 cells were measured, and the size distribution of budded cells in the culture was also measured. Microscopic measurement was used to calculate cell/

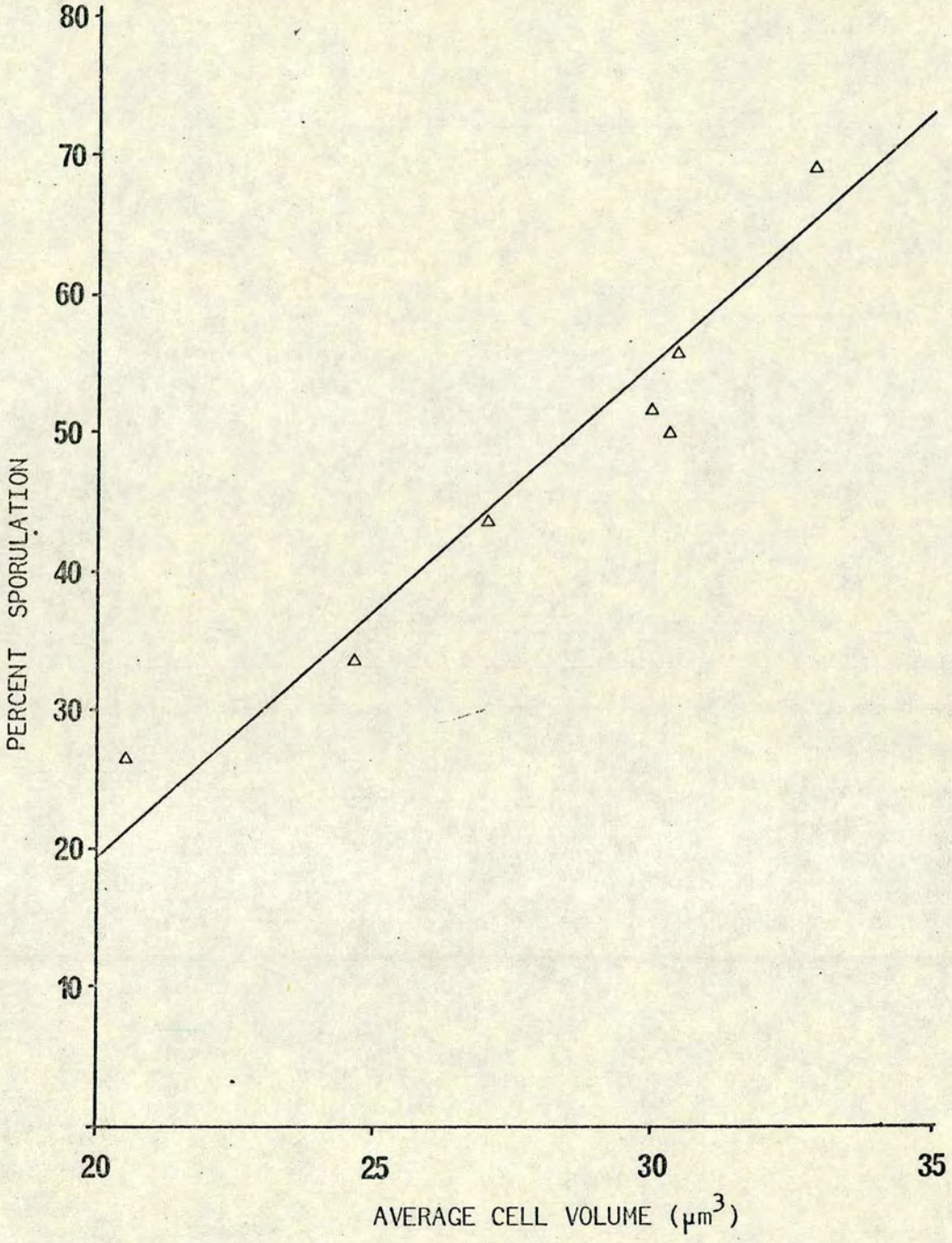



Figure 6.6 Relationship between the percentage sporulation of seven chemostat-grown cultures on resuspension in KAc, and their average cell volume at the time of resuspension.

/volumes, rather than automated methods, for two reasons. Firstly, it was essential to be able to measure cell volumes independently of whether the cells were budded or unbudded, or possibly in small clumps. Second, to obtain the size distribution of budded cells in the culture, it was essential to be able to make distinct measurements of budded and unbudded cells.

The data obtained were first used to make an estimate of the critical size. This was obtained by plotting the frequency of axi against average cell size (Figure 6.6). It was assumed that when the average cell volume was equal to the critical volume, half the cells would be above this volume, and half below it, and that the sporulation frequency would therefore be half the maximum obtainable. Since the maximum sporulation frequency was 80%, 40% was chosen as the frequency corresponding to the critical size, and from Figure 6.6, this gave a critical cell volume of $26\mu m^3$.

Figure 6.7 shows the relationship of the sporulation frequency of the cultures to the percentage of cells in the chemostat culture (immediately before initiation) that were larger than $26\mu m^3$ in volume. Again the relationship was one of direct proportionality, and the two variables were almost equivalent. This result was entirely consistent with there being a critical cell volume of $26\mu m^3$ below which cells will not sporulate. This will be examined further in the discussion to this chapter.

Figure 6.8 shows an average size distribution for budded cells; the results were taken from several experiments, the dilution rate in all cases being $0.0478h^{-1}$. The bimodal shape of the distribution was due to the heterogeneity of the population which is composed of both large mother cells and small daughter cells. The significance of this lies in the fact that 35% of the cells were below the critical size for sporulation, but were still able to initiate budding, indicating that the cell division cycle size control and the sporulation size control were operating at different sizes and are therefore probably independent.

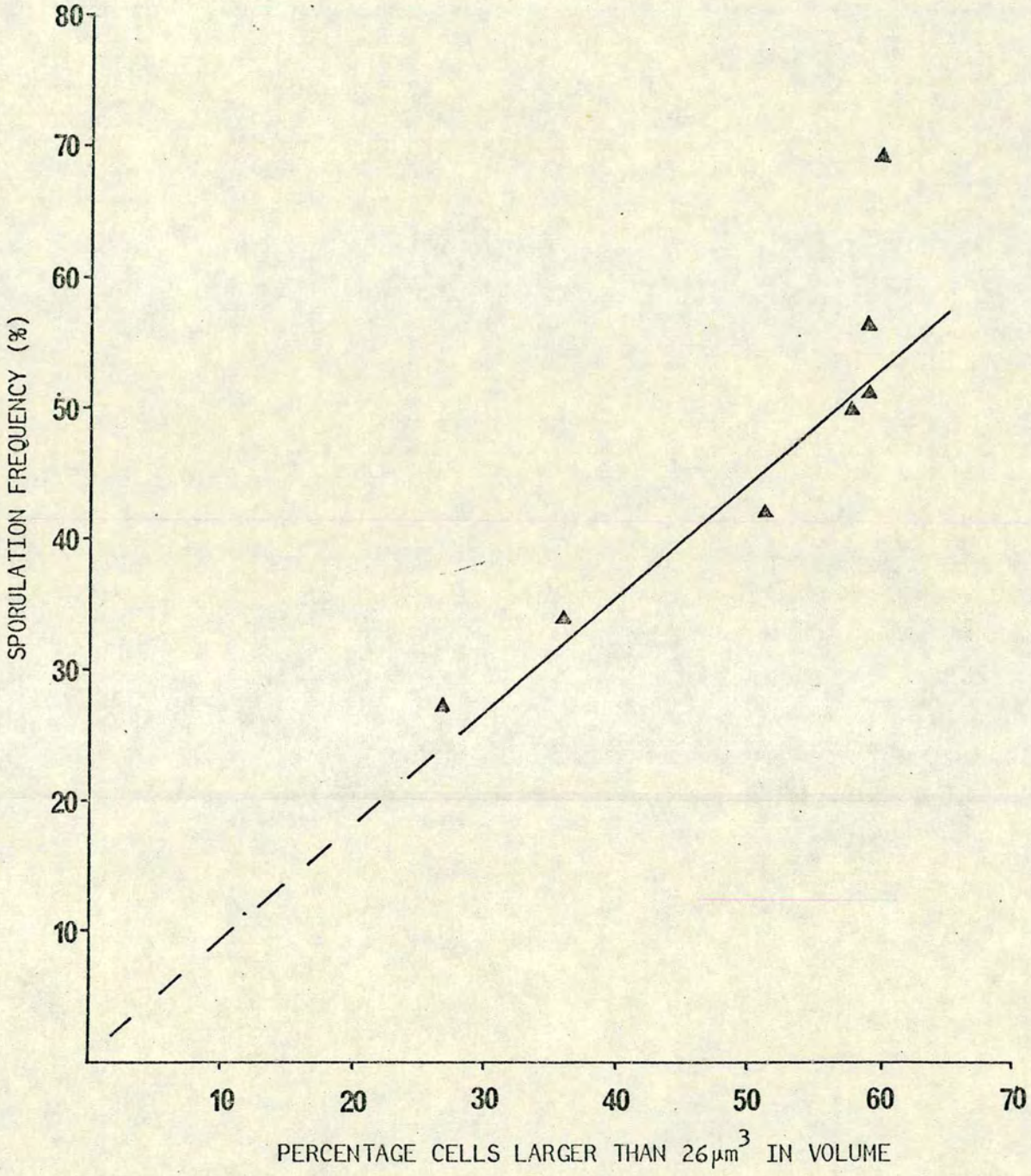
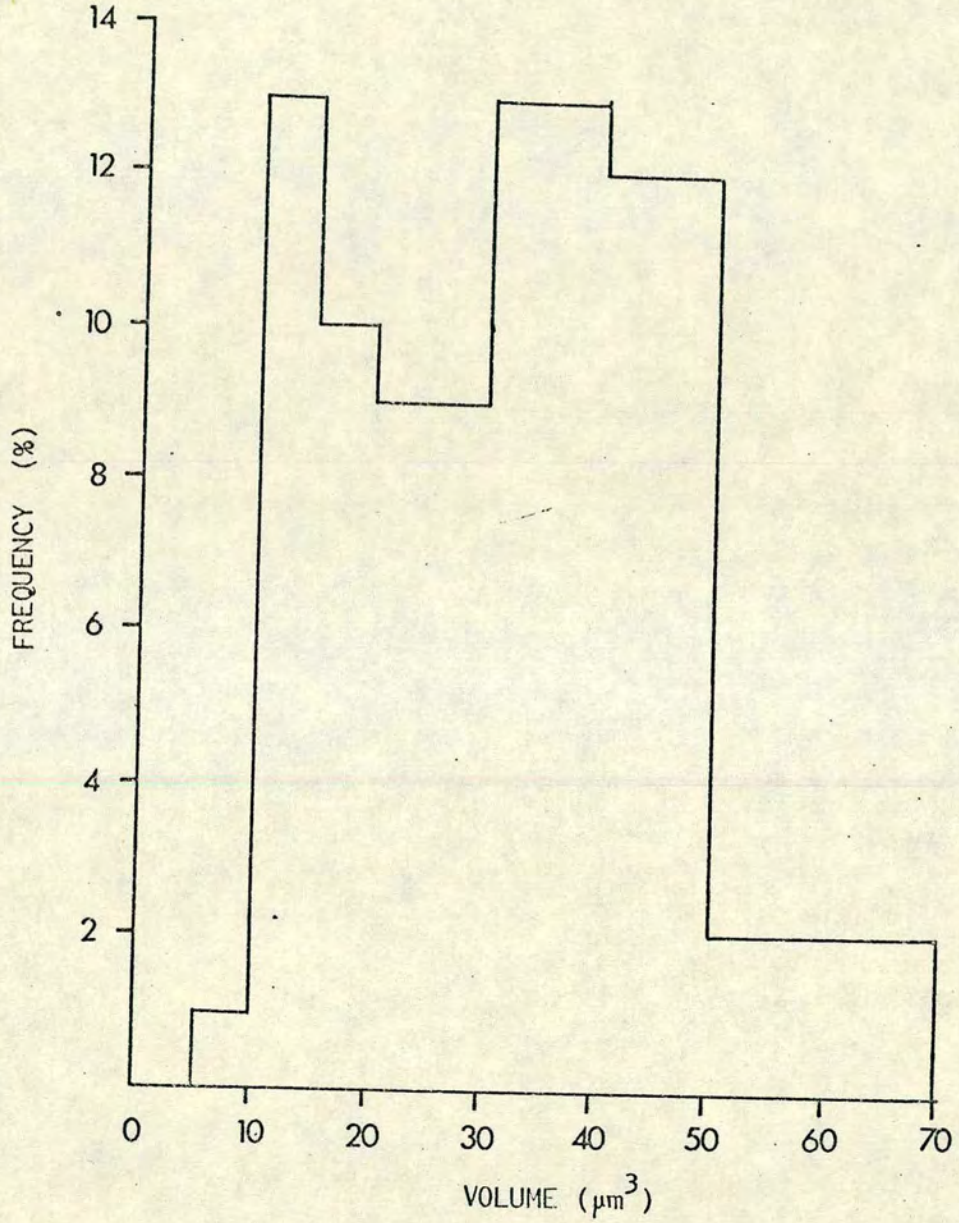



Figure 6.7 Correlation between the percentage of cells larger than $26\mu m^3$ in volume in a chemostat culture, and the percentage sporulation on subsequent resuspension in sporulation medium.




Parent of budded cells

Figure 6.8 Distribution of cell volumes in a chemostat culture growing at a dilution rate of 0.0478($h^{-1}$) (Average of 3 measurements).

## Discussion

Nutrient Limitation Comparing carbon and nitrogen limitation, it is clear that the former is much more effective in inducing cells to initiate sporulation, since the overall sporulation rates achieved under nitrogen limitation were much lower, for a given dilution rate, than those obtained under carbon limitation. Under carbon limitation, the differences between the spd1 mutant and the wild-type were quantitative rather than qualitative, in that the mutant was much more sensitive to carbon source levels, and appeared to initiate sporulation at carbon source levels under which the wild-type continued growth. In relation to nitrogen limitations, the spd1 mutant was again much more sensitive in its response to nitrogen starvation than was the wild-type. The data from the nitrogen limitation experiments gave important evidence for the nature of control over sporulation. The fact that it is possible to obtain cultures sporulating in steady state shows that initiation involves an element of chance. Since all cells were under the same nutrient conditions, and some sporulated whereas others did not, nutrient conditions cannot have absolutely determined the decision to sporulate, but they only influenced the probability of it taking place.

The data of Figure 6.4 fits with a probabilistic model, since an increase in dilution rate would lead to a reduction in generation time, and the cells would spend less time in the 'window' in the cell cycle during which sporulation could be initiated and would be less likely to do so. A possible explanation for the difference in behaviour between S41 and the spd1 mutant under nitrogen limitation is that it is due to the loss of nitrogen repression of sporulation in the latter strain (p.40 ).

In an S41 culture, the initiation rate would be reduced as the dilution rate rose by two factors, the 'window' effect described above, and the changed nutrient conditions. Under double repression, its initiation rate would quickly fall as the dilution rate rose. Strain 59-4A, however, which has lost nitrogen repression of/

/sporulation and is therefore subject only to the 'window' effect, would be less sensitive to the rise in dilution rate, as was observed. To prove that this was the case would require much further study involving measurement of nutrient levels, cell cycle lengths, and lengths of phases of the cell cycle. It should also be noted that the carbon substrate used in these experiements, galactose, is fermentable, and therefore the increased sporulation of the spd1 mutant was being expressed on a fermentable substrate whereas it is normally expressed only on non-fermentable carbon substrates.

As described in Chapter 4, the asporogenous mutant which arose in the chemostat run shown in Figure 6.1 was found to carry a mutation which failed to complement spo53. Otherwise its behaviour was the same as the other spo0 mutants. This is interesting since this mutant was isolated directly from the wild type as an asporogenous initiation mutant, rather than as a revertant from an spd1 strain, which is how the others were isolated. As the selective advantage for this mutant in the chemostat lies in its ability to continue vegetative growth when the wild-type sporulates, its method of isolation identifies it as an initiation mutant, and indicates that spo53 and, therefore, presumably, spo50 and spo51, are initiation mutations.

Cell size control over Sporulation The evidence presented in this Chapter indicates that sporulation is controlled according to the cell size, and that this control operates at a size larger than that operating over cell division. The minimum size for budding in the conditions tested was between 5 and 10$\mu m^3$, whereas the minimum size for sporulation was 26$\mu m^3$. Thus 35% of cells able to commence budding were too small to sporulate.

Models for size control are more difficult to establish. In regard to size control over cell cycle initiation, a deterministic inhibitor dilution model (Ycas et al., 1965; Fantes et al., 1974) was developed, in which an unstable inhibitor is produced in the cell at a constant rate, and gradually diluted as the cell increases in/

/size, until a critical concentration is reached, at which the cell is able to initiate a cycle of cell division.

The early evidence on size control (Johnston et al., 1977, 1979; Lorincz & Carter, 1972) in Saccharomyces cerevisiae indicated that there was a critical size absolutely determining initiation of the cell cycle. Wheals (1982) has presented good evidence for a probabilistic element in size control, whereby cell size does not absolutely determine whether initiation can take place, but determines the probability of it taking place. In this type of model, there would be a range of cell sizes over which the probability of initiation of sporulation increased gradually from 0 to 1.0. Depending on the shape of the curve describing the change in probability, the 'critical size' observed previously would reflect a cell size somewhere near the 0.5 probability point in the curve. The extent to which the size control appeared to be deterministic would then reflect the sharpness of the transition from 0 to 1.0 probability, i.e. the range of sizes over which the transition took place.

In relation to sporulation, our evidence cannot distinguish between probabilistic and deterministic models for cell size control, as the evidence only relates to populations and not individual cells. To make the distinction would involve observing individual cells and the size at which they initiate sporulation. It is likely, however, that cells use a similar mechanism to control sporulation by cell size as they do to control cell cycle initiation. It is also possible that the cell uses the same control system for both, but that the initiation of sporulation is more sensitive to the proposed inhibitor, and it therefore needs to be diluted more to enable initiation of sporulation. The evidence from the whi mutants has some bearing on this. These mutants are altered in the size at which they can initiate buds (Sudbery et al., 1980) but not, apparently, in the critical size at which they can initiate sporulation. If they are affected in the production of an unstable inhibitor, this would indicate that cell cycle initiation and sporulation are not controlled by the same inhibitor. If they are/

/affected in the cell cycle response to the inhibitor, both could still be under control by the same inhibitor.

It is also possible that the correlation between size and ability to sporulate does not reflect a direct control over sporulation by cell size, but that the cell size affects some aspect of the physiology or nutritional status of the cell which in its turn influences sporulation. Coordinate control is common in yeast (See the Sections on nitrogen and carbon source regulation in the introduction), and cell size is likely to have a direct effect on nutritional status as it obviously determines the amount of cytoplasm, and therefore metabolic machinery, available to the control of a particular nucleus.

Cell size will also determine to some extent the amount of mitochondrial and other energy-yielding activities available for the processes of sporulation in a particular cell, and also the reserves of structural components and energy stores. It is clear that were a cell to commence sporulation at a point when it did not have either enough of the structural monomers needed, or the capacity to produce enough energy, to complete the process, it would fail to produce mature spores and this would presumably result in cell death. The correlation between ability to sporulate and cell size is presumably the cell's mechanism to avoid this occurring. Since sporulation is itself a survival response to starvation, it is especially important that the status of the cell should enable it to complete the process.

Possible ways of extending this work are examined in Chapter 8.

# CHAPTER 7

## NEW GENE EXPRESSION DURING SPORULATION

### INTRODUCTION

The aim of the work presented in this chapter was to establish whether there were any polypeptides produced de novo during sporulation and which were specific to the process. The most obvious method of doing this would be by pulse labelling with radio-labelled amino acids followed by extraction and separation of labelled proteins. Initial attempts to do this by pulse-labelling proteins and separating them by one-dimensional (Hopper et al., 1974) and two-dimensional (Trew et al., 1979) polyacrylamide gel electrophoresis failed to demonstrate the synthesis of any new sporulation-specific proteins. This result was surprising since it indicates that sporulation, a morphogenetic process involving considerable changes in structure and physiology occurs without any major protein being produced de novo. Dawes et al. (1980) showed, however, that the failure to detect any new protein synthesis by pulse-labelling actually reflected a defect inherent in the method. Cells later on in the sporulation process failed to adequately take up amino acids, and therefore in a pulse-labelling experiment, most of the label was being taken up by non-sporulating cells and cells early in sporulation.

The use of pre-labelling, that is labelling cells in the vegetative growth phase and then following the status of the labelled proteins during sporulation has indicated that a large number of individual polypeptides change in sporulating cells (Wright & Dawes, 1979; Peterson et al., 1979) and that these changes are specifically timed (Wright et al., 1981; Ajam et al., 1981). These changes include both appearances, disappearances and changes in concentration.

Unfortunately, methods involving uniform labelling of all cell polypeptides followed by separation and detection make it difficult to assess the contribution of new gene expression to/

/these changes, although indications of cycloheximide dependence have been found in some of the changes observed (Ajam, 1981).

It is important to assess the contribution of new gene expression during sporulation in order to discover the mechanisms controlling meiosis and sporulation. Obviously, if there is little new gene expression during sporulation, it is futile to look for controls at the level of transcription. On the other hand, the presence of new gene expression indicates a direct genetic control at the level of transcription.

The work presented in this chapter was designed to detect new gene expression by assaying for new messenger RNA species in the sporulating cells. The appearance of new mRNA species can be detected by extracting the mRNAs from cells at various stages in the sporulation process, and by using them in an *in vitro* translation system, to obtain their polypeptide products. These can be visualized by incorporating labelled amino acids during translation and by separating and detecting the synthesised polypeptides in a polyacrylamide gel electrophoretic system.

The *in vitro* translation system used was a rabbit reticulocyte lysate (Hunt & Jackson, 1974; Pelham & Jackson, 1976; Villa-Komaroff *et al.*, 1974). This system was prepared by enriching rabbit blood *in vivo* for reticulocytes, which are the immature erythrocytes with extremely active protein synthesis. A lysate was prepared from the blood and treated with a calcium-dependent micrococcal nuclease to destroy endogenous message. The nuclease was then inactivated by the addition of a calcium chelating agent. The treated protein synthesising system could then translate exogenous mRNA and incorporate labelled amino acids into the products.

## Development and Characterization of a Yeast mRNA-primed *in vitro* Translation System

Since *in vitro* translation systems vary in their characteristics according to their source and the type of message used, the first/

| CONDITIONS | Rate of ($^{3}$H) phenylalanine incorporation | | Rate of ($^{35}$S) Methionine incorporation | |
|---|---|---|---|---|
| | COUNTS * $M^{-1}$ | INDEX | COUNTS * $M^{-1}$ | INDEX |
| Optimal System | 2,000 | 1.0 | 52,000 | 1.0 |
| Addition of ATP/GTP | | | 1,040 | 0.02 |
| Incubation without added RNA | 100 | 0.05 | 2,600 | 0.05 |
| " " creatine phosphate | | | 21,840 | 0.42 |
| " " salts/DTT | | | 24,440 | 0.47 |
| " " spermidine | | | 39,520 | 0.76 |
| " " creatine phosphokinase | | | 41,600 | 0.80 |
| Addition of amino acids | 2,000 | 1.0 | 52,000 | 1.0 |
| 0.40% dilution with salts | 2,000 | 1.0 | 52,000 | 1.0 |

Table 7.1 Characteristics of yeast mRNA- directed *in vitro* translation by an mRNA-dependent rabbit reticulocyte lysate.

* units are: counts $min^{-1}$ $ml^{-1}$ of lysate, measured over 30 min.

| CONDITIONS | Rate of ($^{3}$H) phenylalanine incorporation | | Rate of ($^{35}$S) methionine incorporation | |
|---|---|---|---|---|
| | COUNTS* MIN | INDEX | COUNTS* MIN | INDEX |
| a) Translation on endogenous message | 9,500 | 1 | 249,600 | 1 |
| b) Translation after nuclease treatment | 100 | 0.011 | 1,040 | 0.0042 |
| c) Translation after addition simultaneously of nuclease and EGTA | 9,500 | 1 | 249,600 | 1 |

Table 7.2 Table showing a) rate of translation by an untreated lysate, b) rate of translation by a nuclease-treated lysate, and c) rate of translation by a lysate treated with inactiviated nuclease.

* units are: counts $min^{-1}$ $(ml\ lysate)^{-1}$ measured over 30 min

/step was to obtain a system and characterize its properties using yeast mRNA. Table 7.1 shows the effect of addition or removal of several components of the rabbit reticulocyte system deduced to be necessary by Villa-Komaroff et al. (1974) and Pelham and Jackson (1976). The reference system indicated by "optimum system" was that described in the materials and methods section, and used in later experiments. Points to be noted were that ATP and GTP, added at $3.6mgml^{-1}$ and $0.72mgml^{-1}$ respectively, were severely inhibitory to the system. Both ATP and GTP were necessary additions to the systems of Villa-Komaroff et al. (1974) and Pelham and Jackson (1976). Presumably, this system already contained enough ATP and GTP to provide the initial energy supply and to provide enough ADP and GDP to the energy yielding creatine phosphate/creatine phosphokinase to be recycled into ATP and GTP in order to continue energy supply to the translation apparatus. Also, addition of exogenous amino acids made no difference at all to the activity, although these too were found to be essential in previous systems. Removal of creatine phosphokinase did not severely inhibit incorporation in the first 30m., but incorporation was more severely affected after that, presumably as the system failed to resynthesize ATP for energy.

The background incorporation without exogenous mRNA was low, at about 5% of the optimum level. In fact, in the optimum system, total cell RNA was used, without enriching for polyadenylated RNA, as many eucaryotic mRNAs are not polyadenylated (Katinakis et al., 1980) and any such messengers in the yeast cells would not have been detected if the system had been primed with RNA enriched for polyadenylated species.

Finally, it was found that, as long as pH and salt concentrations were kept constant, the lysate could be diluted to 60% of its normal strength without reducing incorporation. This was important in establishing how much mRNA and labelled amino acids could be added.

Table 7.2 shows the results of experiments on the efficiency of nuclease treatment. Nuclease treatment for 15min, followed by/

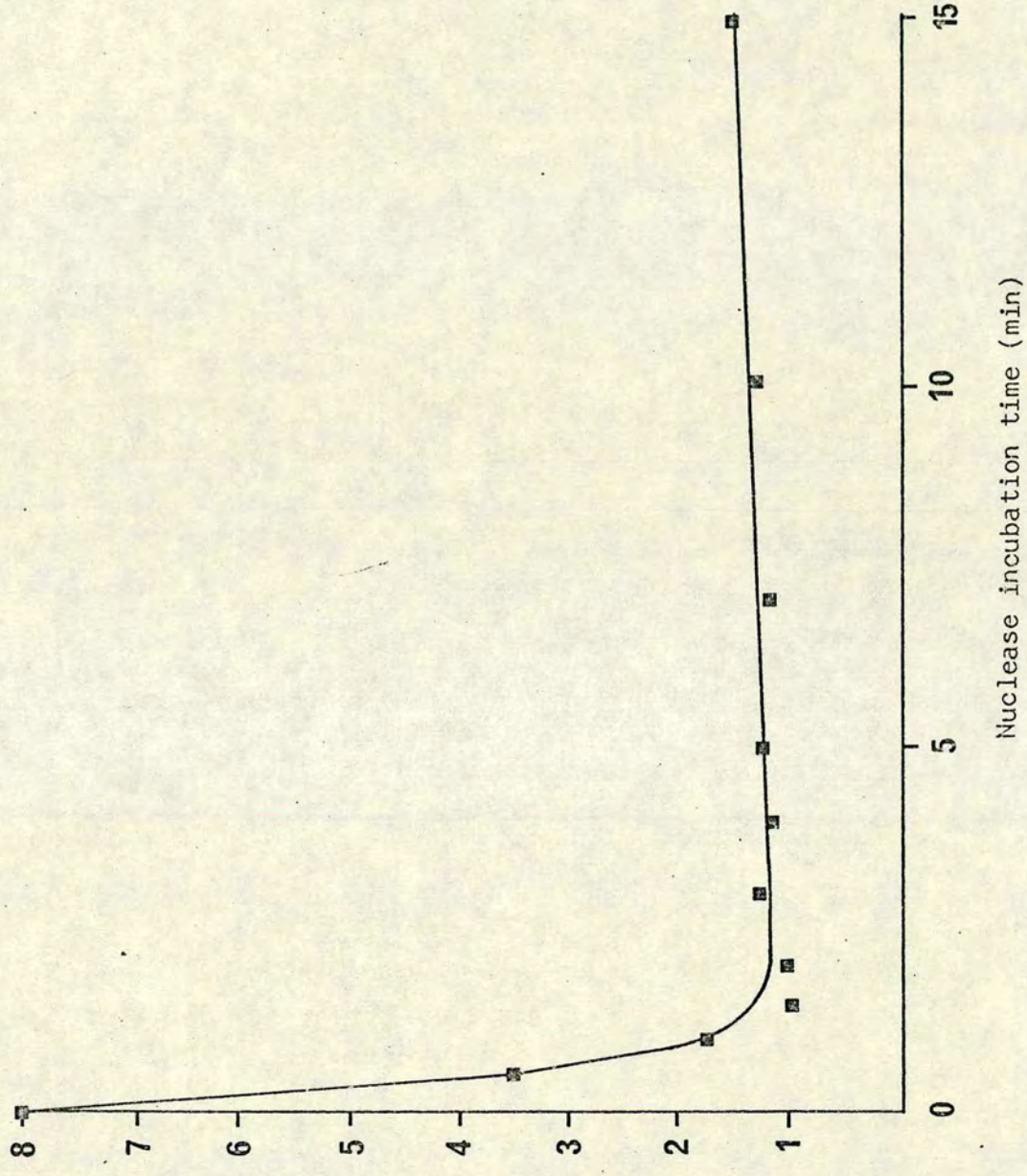



Figure 7.1 Graph showing reduction in endogenous amino-acid incorporating ability by a rabbit reticulocyte lysate during nuclease treatment

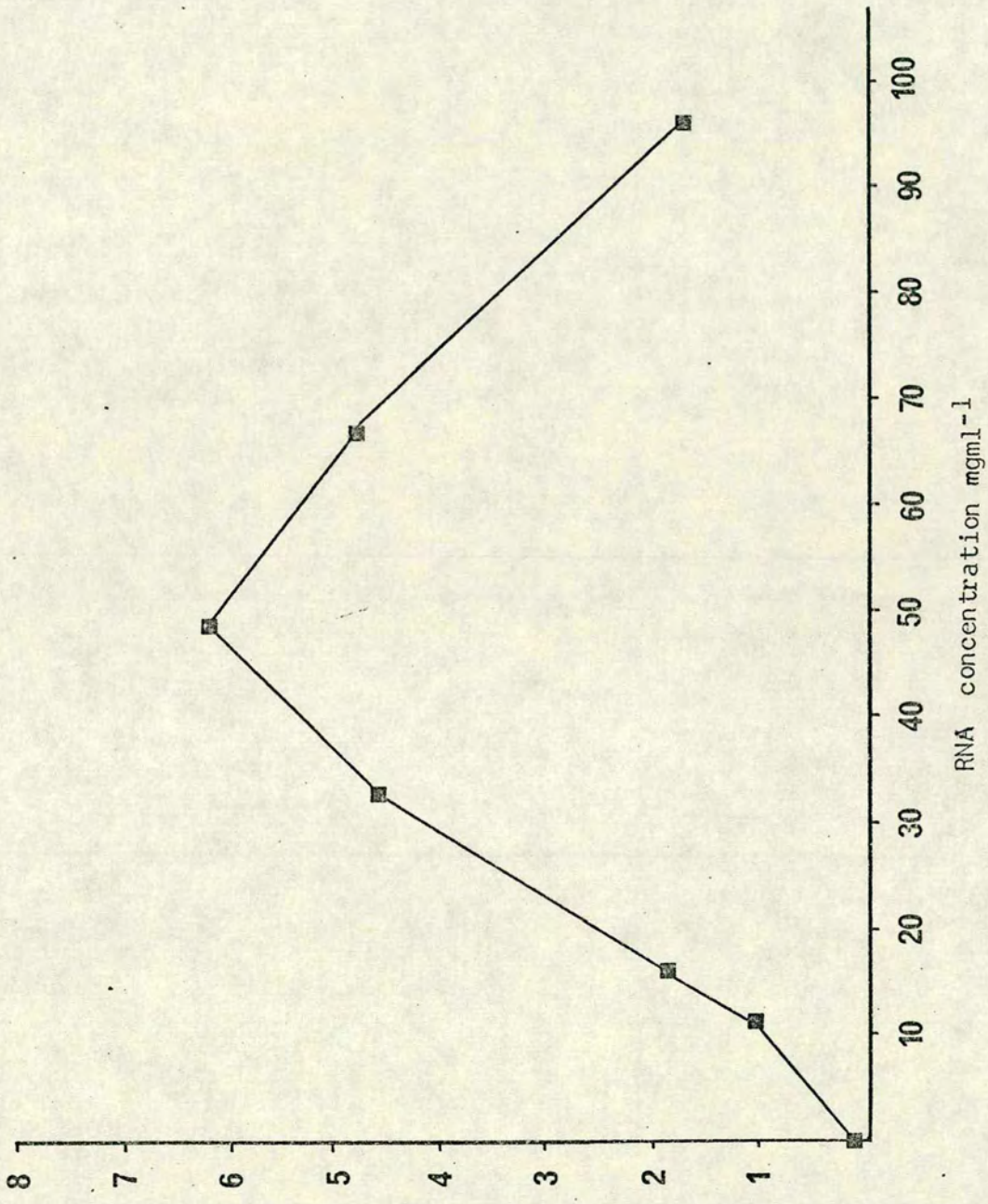



Figure 7.2 relationship between amino-acid incorporating ability in an mRNA-dependent rabbit reticulocyte lysate and the concentration of added mRNA.

/the addition of EGTA abolished over 48% of the system's ability to incorporate using endogenous message as template. Immediate inactivation of the nuclease by EGTA left the system with 100% of its incorporating activity, indicating that neither EGTA nor inactivated nuclease was inhibitory to the system.

Figure 7.1 shows the time course of nuclease inactivation of the endogenous level of incorporation. $CaCl_2$ and nuclease were added to the system, and samples were taken at various intervals, EGTA was added, and the sample tested for endogenous incorporating activity. As can be seen, the 15min normally allowed for nuclease treatment was quite adequate, as endogenous activity reached a minimum after 2min.

Finally, Figure 7.2 shows the dependence of ($^{35}S$) incorporating activity on RNA concentration. The RNA used was the total cellular RNA extracted from sporulating cells, and was that used in the experiments described in the following two sections.

Polyacrylamide Gel Electrophoresis of Tritium-labelled in vitro Translation Products

The above experiments having shown that the rabbit reticulocyte lysate was able to incorporate amino acids into polypeptide material using yeast mRNA as template, the following experiment was performed to test whether the polypeptides being synthesized were of high molecular mass and not simply short, low molecular mass fragments. Before going on to label polypeptides at high activity using ($^{35}S$) -methionine in order to detect individual species, this preliminary test was needed to establish whether these large polypeptides were maintaining their integrity through the incubation and separation processes.

Two reticulocyte lysate aliquots were incubated with L-($^{3}H$) phenylalanine, as described in the materials and methods section, one with 0hr sporulation RNA (total) and one with the equivalent volume of distilled water. After incorporation, samples containing 100,000 counts $min^{-1}$ of TCA-insoluble material, and equivalent volumes of/

Figure 7.3 Profile of incorporated activity ($^3H$) activity and protein levels along a polyacrylamide gel after electrophoresis of rabbit reticulocyte lysate samples incubated with yeast RNA. Single line: incorporation of ($^3H$) phenylalanine; Shaded area: absorbance at 280nm; Hatched area: incorporation of ($^3H$) phenylalanine by control lysate with added message.

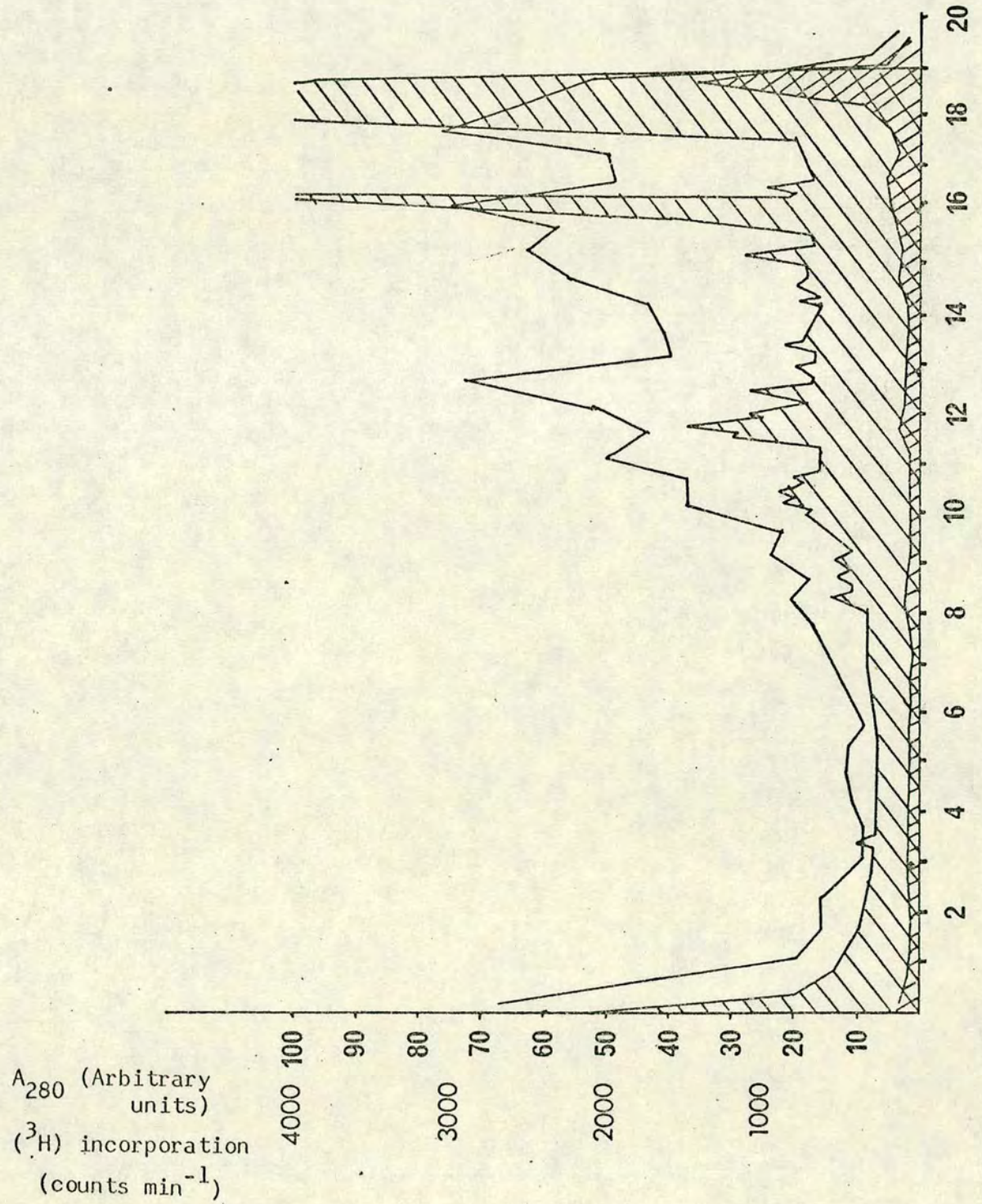
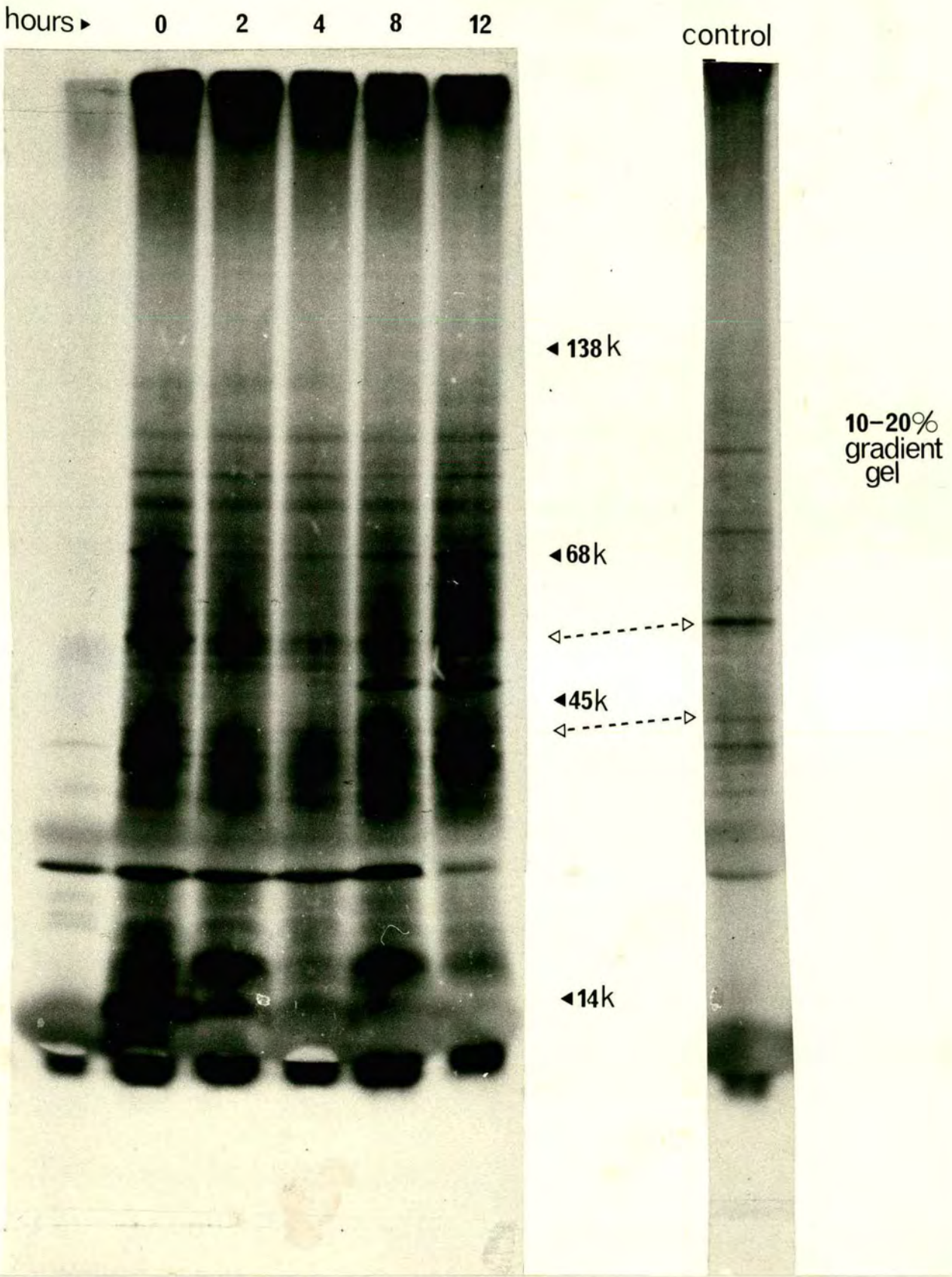


Figure 7.4 (Opposite)

Autofluorograph of a polyacrylamide gel after electrophoresis of rabbit reticulocyte lysate samples incubated with RNA extracted from sporulating yeast and translated in the presence of ($^{35}$S) - methionine. Tracks are, left to right, first a lysate incubated without added message, second (0) a lysate incubated with RNA from vegetative cells, (2) (4) (8) and (12) lysates incubated with RNA from cells 2, 4, 8 and 12h into sporulation respectively, and (CONTROL), a lysate incubated with RNA from non-sporulating a/α cells exposed to sporulation medium for 12h. Solid arrows indicate positions of molecular mass markers. Dotted lines and open arrows link corresponding bands on control and test gels.


hours ▸
0
2
4
8
12
control
◂ 138 k
◂ 68 k
◂ 45 k
◂ 14 k
10–20% gradient gel

Polyacrylamide Gel Electrophoresis of sulphur -35 labelled in vitro Translation Products

In this case seven incubations were performed with the reticulocyte lysate, each containing ($^{35}$S)-methionine at 1 li $l^{-1}$ and either a test RNA at 50 $gml^{-1}$ or the equivalent amount of distilled water. The six test RNA's used were total RNA extracted from sporulating cells 0, 2, 4, 8 and 12h after initiation, and total RNA extracted from non-sporulating ∝/∝ cells after 12h after initiation. The seventh incubation was a control with no exogenous RNA. After translation, the samples were electrophoresed on an SDS-polyacrylamide gel system, together with four molecular mass standards [illegible]. After electrophoresis the gels were stained with Coomassie blue and the ($^{35}$S)-labelled protein bands were detected by autofluorography. Figure 7.4 shows the final autofluorograph together with the ∝/∝ control track run on a separate gel, the positions of the molecular mass standards, and dotted lines linking equivalent bands on the test RNA tracks and the ∝/∝ non-sporulating control.

It can be seen clearly that there was a single heavy band, corresponding to a protein with a molecular mass in the range 49,000D-51,000D, which appeared in the eight hour sample but not in the four, two or zero hour samples. This protein did not appear in the non-sporulating ∝/∝ control sample and is therefore presumably sporulation-specific. There were also a number of other changes which were visible on the original autofluorograph but are not visible on the photograph. None of these other changes could be confirmed as sporulation-specific. It must be emphasized that, although the appearance of a 50,000m protein was detected on the gel, one was actually assaying the appearance of a new translatable mRNA species in the sporulating cells.

Discussion

The evidence presented in this chapter has demonstrated the/

/specific appearance in sporulating cells only, of at least one abundant mRNA species during sporulation, presumably indicating that its corresponding polypeptide is synthesized de novo. The abundance of the major new mRNA species, as shown by the prominence of the translated protein band, indicates that the corresponding polypeptide was a major component of the cell, and it may therefore be a structural protein. A possible candidate for the de novo synthesized protein is the spore coat surface protein detected by Briley et al. (1970). This protein appears to be unique to sporulation and is synthesized in large amounts late in the process. The mRNA species we have detected appeared some time before the time of appearance of this spore coat surface protein. It is possible however that the message for this protein appears early in sporulation but is not translated in vivo till later. It is also possible that the protein is synthesized early but not deposited on the spore surface till later.

Figure 7.4, showing the results of autofluorography, also shows the major limitation of this method, which is the small number of different polypeptides which can be detected. Weir et al. (1982) have recently greatly extended the method by using two-dimensional PAGE and autofluorography to resolve polypeptides translated in vitro from mRNA extracted from sporulating cells. Using a fuller time course of sporulation from 0-24hr, and the corresponding full time course for non-sporulating a/a and α/α cells, they were able to confirm the sporulation-specific appearance of the 50,000 D translation product, faintly after 6h and fully after 8h. Using the fuller resolution obtainable by two-dimensional PAGE, they were able to show many other changes in translatable RNA during sporulation, many of which were also sporulation-specific.

The problem in using these techniques to study sporulation lies in the difficulty of associating bands or spots appearing on polyacrylamide gels with a particular functional cell protein. However, if several possible identities for a particular protein are suspected, immunoprecipitation methods can be used to confirm the identity of a particular translation product. A general conclusion can be made, however, that de novo synthesis of proteins is important in/

/sporulation, as shown by the number of new mRNA species appearing during the process. The methods used only detected abundant mRNA species, and so are unlikely to detect regulatory gene products, but the specific timing apparent in the appearance both of new proteins and new mRNAs indicates a tight regulation over the pattern of protein synthesis and modification, and therefore a degree of genetic control over sporulation. This conclusion is rather conjectured, but is supported by a great deal of other evidence for the importance of gene expression and genetic control over sporulation, particularly that of Esposito & Esposito (1979, 1974b). At present, however, it still remains difficult to establish which genes have a regulatory function and which are essential to sporulation.

## CHAPTER 8 - GENERAL DISCUSSION

The results presented in this thesis have identified several controls over sporulation, including (i) a negative control over the initiation of sporulation exercised by the genes mutated in spd1 and spd3 mutants, which acts independently of other initiation genes, (ii) a positive control over initiation exercised by the genes mutated in spo50, spo51 and spo53 mutants, also independent of other initiation genes, (iii) a chance element in nutritional control, as opposed to an all-or-nothing control, (iv) a cell-size control over the initiation of sporulation, and (v) a control over gene expression during the process, subsequent to initiation.

The first two controls, exercised by the genes mutated in spd and spo0 mutants, are closely related to the capacity of the cell to carry out respiration. A major conclusion of interest from this is that the effect of respiratory capability on sporulation is not a direct one, as the relationship between respiratory capability and ability to sporulate in these mutants is the opposite to that which would be expected if sporulation ability were simply dependent on respiratory capability. The evidence presented in Chapter 5 does indicate some connection between mitochondrial biogenesis and activity, and sporulation ability. Since mutants derepressed for mitochondrial biogenesis are available (Boker-Schmitt et al., 1982) which resemble the spo0 mutants in their tetrazolium reduction characteristic, an obvious further step would be to test these for their sporulation ability, and their ability to suppress the spd1, spd3 and spd4 mutations, and also to test the spo0 mutants directly for any change in mitochondrial biogenesis or activity which could account for the change in sporulation capability. Simple assays for mitochondrially-located enzymes should detect any central metabolic defect in the spd or spo0 mutants, and could also detect activities of these enzymes present in these mutants when they would normally be repressed. It is possible that the spo0 mutants are not blocked at the point of initiation of sporulation, but complete some early sporulation events before they are blocked. /

/Tests for important early sporulation events such as premeiotic DNA synthesis and intragenic and intergenic recombination in spo0 mutants introduced into sporulation medium should establish whether they are blocked in initiation. A powerful technique for tracing how substrates are used by yeast cells, involving spin-labelling with $^{13}C$ compounds and nucleomagnetic resonance spectroscopy (J R Dickinson, pers. comm.) can now be used to find any alteration in uptake and utilization patterns in such mutants as those strains carrying the spd and spo0 mutations, which have altered growth on certain substrates.

Evidence from continuous cultivation studies indicates that yeast cells are much more sensitive to carbon-source regulation. Nutritional control could be of two sorts, (i) a type of control in which there is an all-or-nothing response according to whether the nutrient level is over or under a certain threshhold value, and (ii) a type of control in which the probability of initiation is variable between 0 and 1, and dependent on nutrient level. In fact, nutritional control over sporulation appears to contain elements of both types of control. Further work using continuous cultivation techniques would involve direct measurement, in both carbon and nitrogen limited chemostat cultures, of limiting nutrient levels, dissolved oxygen levels, specific growth rates and sporulation initiation rates, from which it should be possible to determine which of these are directly dependent on each other, and what the kinetics of the regulatory mechanisms are.

The cell size control over sporulation could also operate with an all-or-nothing critical size mechanism, or with a mechanism involving varying probability, as the evidence presented here cannot distinguish between these. It seems likely, however, that the cell-size control over sporulation has a similar mechanism to that over the cell division cycle, which appears to have elements of both types of control (Wheals, 1982). In order to define more precisely how the size control over sporulation operates, it would be necessary to follow the behaviour of individual cells by/

/microscopic examination, noting their size at specific times and whether they subsequently complete sporulation. It would, however, be technically difficult to maintain cells under direct microscopic examination over the 20h or so which would be needed. The two size controls, that over the cell division cycle, and that over sporulation may have arisen independently in evolution, or the sporulation size control may have developed from the other, the selective pressure being exerted by the cell death associated with abortive sporulation of cells large enough to escape the control over cell division but lacking sufficient resources to complete sporulation.

The work presented in Chapter 7 showed that there was new gene expression during sporulation and, therefore, control of the process at the level of transcription. Extension of this work (Weir et al., 1982) has shown that a number of mRNA species coding for corresponding polypeptide species are produced de novo during sporulation in a timed sequence reminiscent of that observed in experiments on in vivo polypeptide synthesis seen with pre-labelled cells (Ajam et al., 1981). Some of the changes in individual polypeptide species observed by Ajam et al.(1981) were, therefore, presumably in polypeptides shown to be produced de novo by Weir et al. (1982) and the timed sequence of changes in cell polypeptide species observed in the former study probably reflected, at least in part, an orderly timed sequence of gene expression. There are indications from all these studies, therefore, that the events of sporulation are under close control at the level of transcription.

The most obvious further step in examining the control of gene expression would be to attempt identification of the proteins responsible for some of the spots observed on the polyacrylamide gels. If an intelligent guess could be made about the identity of some of these polypeptides, antibodies could be raised against the suggested protein, and by an immunoprecipitation method, the protein could be identified on a gel. Alternatively, to identify/

/the product of a particular gene on a gel, it would be possible to obtain cloned fragments of DNA containing the particular gene and isolate the complementary RNA which could then be used as a message to prime an *in vitro* translation system to synthesize the relevant protein. Alternatively, if a cloned DNA fragment were available, it could be hybridized to the RNA in the sample to be used for *in vitro* translation, and when the translation products were electrophoresed and visualized, the product corresponding to the complementary RNA to the DNA used would be missing and could therefore be identified on a control gel.

All the above methods could be used to remedy the inherent problem in electrophoretic methods, which is that it is difficult to correlate a change in a spot on a polyacrylamide gel with a change in a particular cell protein.

In conclusion, the evidence presented in this thesis has gone some way towards the ultimate goal, the full elucidation of all the factors affecting or regulating the initiation and progress of sporulation in *Saccharomyces cerevisiae*, however a great deal of further work is necessary before this goal can be fully realized.

REFERENCES

Adams, J., (1977), Exp. Cell Res. 106: 267 - 275

Ajam, N., Wright, J.F., and Dawes, I.W., (1981) Curr. Dev. Yeast. Res. 5: 271 - 275

Arst, H.N. and Cove, D.J. (1974) Mol. Gen. Genet. 126: 111 - 141

Beck, L. and von Meyenburg, H.K., (1968) J. Bact. 96: 479 - 486

Bedard, D.P., Johnston, G.L. and Singer, R.A. (1981) Curr. Genetics 4: 205 - 214

Betz, H., (1979) F.E.B.S. Letts. 100: 171 - 174

Boker-Schmitt, E., Francisci, J. and Schweyen, R.J., (1982) J. Bact. 151: 303 - 310

Bossinger, J., Lauther, R.P. and Cooper, J.G., (1974) J. Bact. 118: 821 - 829

Briley, M.S., Illingworth, R.F., Rose, A.H., and Fisher, D.J.(1970) J. Bact. 104: 588 - 589

Bucking-Throm, E., Duntz, W., Hartwell, L.H. and Manney, T.R. (19739 Exptl. Cell Res. 73: 99 - 110

Bugeja, V.C. and Carter, B.L.A. (1982) J. Bact. 151: 1051 - 1052

Byers, B. and Goetsch, L., (1975) Proc. Nat. Acad. Sci. U.S.A. 72: 5056 - 5060

Carter, B.L.A., (1978) Adv. Micro. Physiol. 17: 244 - 297

Carter, B.L.A., and Jagadish, M.N., (1978) Exp. Cell. Res. 112: 373 - 383

Carter, B.L.A. and Sudbery, P.E. (1980) Genetics 96: 561 - 566

Chen, A.W. and Miller, J.J., (1968) Can. J. Microbiol. 14: 957 - 963

Colunna, N.J. and Magee, P.T., (1978) J. Bact. 134: 844 - 853

Cooper, T.G., (1980) Trends in Biochem. Sci. 6: 332 - 334

Coote, J.G. and Mandelstam, J., (1973) J. Bact. 114: 1254 - 1263

Crandall, M., Egel, R. and Mackay, V.L., (1977) Adv. Micro. Physiol. 15: 307 - 419

Croes, A.F., (1966) Exptl. Cell Res. 41: 452 - 468

Croes, A.F., (1967) Planta 76: 219 - 226

Davidow, L.S., Goetsch, L., and Byers, B., (1980) Genetics 94: 581 - 595

Dawes, I.W. and Hardie, I.D., (1975) Mol. Gen. Genet. 131: 281 - 289

Dawes, I.W. and Mandelstam, J., (1970) J. Bact. 103: 529 - 535

Dawes, I.W. and Thornley, J.H.M., (1970) J. Gen. Microbiol. 62: 49 - 66

Dawes, I.W., (1975) Nature 255: 707 - 708

Dawes, I.W., Wright, J.F., Vezinhet, F. and Ajam, N., (1980) J. Gen. Microbiol. 119: 165 - 171

Del-Rey, F., Santos, T., Garcia-Acha, I. and Nombela, C., (1980) J. Bact. 143: 621 - 627

Dion, P. and Mandelstam, J., (1980) J. Bact. 141: 766 - 792

Donaldson, S., (1977) Thesis for BSc (Hons), Edinburgh University

Dubois, E., Grenson, M. and Wiame, J.M. (1973) Biochem. Biophys. Res. Comm. 50: 967 - 972

Edwards, D.L., Kwiecinski, F., and Horstmann, J., (1973) J. Bact. 114: 164 - 168

Ephrussi, B., and Hottinguer, H., (1951) Cold Spring Harbor Symp. Quant. Biol. 16: 75 - 84

Esposito, M.S. and Esposito, R.E., (1969) Genetics 61: 78 - 89

Esposito, M.S., and Esposito, R.E., Arnaud, M. and Halvorson, H.O. (1969) J. Bact. 100: 180 - 186

Esposito, M.S., Esposito, R.E., Arnaud, M. and Halvorson, H.O., (1970) J. Bact. 104: 202 - 210

Esposito, R.E., Frink, N., Berstein, P. and Esposito, M.S., (1972) Mol. Gen. Genet. 114: 241 - 248

Esposito, R.E. and Esposito, M.S., (1974) Proc. Nat. Acad. Sci. U.S.A. 71: 3172 - 3176

Esposito, M.S. and Esposito, R.E., (1974b) Genetics 78: 215 - 225

Esposito, M.S. and Esposito, R.E., (1975) Methods in Cell Biology XI (D Prescott ed.) pp. 303 - 326, London 1975

Esposito, M.S. and Esposito, R.E., (1978) Biol. Cellulaire 33: 93 - 102

Esposito, R.E. and Klapholz, S., (1981) In The Molecular Biology of the Yeast Saccharomyces (Strathern, N., Jones, E.W. and Broach, J.R., Eds.) pp. 211 - 288 Cold Spring Harbor, New York, 1981

Fantes, P., Grant, W.D., Pritchard, R.H., Sudbery, P.E. and Wheals, A.E., (1974) J. Theor. Biol. 50: 213 - 244

Fantes, P., and Nurse, P., (1977) Exp. Cell Res. 107: 377 - 386

Fast, D., (1973) J. Bact. 116: 925 - 930

Fogel, S. and Roth, Rl, (1974) Mol. Gen. Genet. 130: 189 - 201

Fortnagel, P., and Freese, E., (1968) J. Bact. 95: 1431 - 1438

Fowell, R.R., (1969) In The Yeasts Vol I (Rose, A.H. and Harrison, J.H. Eds.) pp. 386 - 453 Academic Press

Fowell, R.R., (1975) In Spores VI (Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Eds.) pp. 124 - 131 Am. Soc. Microbiol. Washington 1975

Freese, E., (1972) Curr. Topics Dev. Biol. 7: 85 - 124

Friis, J. and Roman, H., (1968) Genetics 59: 33 - 36

Gasior, E., Herrera, F., Sadnik, I., McLaughlin, C.S. and Moldave, K., (1979) J. Biol. Chem. 254: 3965 - 3969

Gasior, E., Herrera, F., McLaughlin, C.S. and Moldave, K., (1979) J. Biol. Chem. 254: 3970 - 3976

Gerlach, W.L., (1974) Heredity 31: 241 - 249

Gillie, O.J., (1970) J. Gen. Microbiol. 61: 379 - 395

Gosling, J.P., and Duggan, P.F., (1969) Biochem. J. 115: 8P

Gosling, J.P., and Duggan, P.F.,(1971) J. Bact. 106: 908 - 914

Grenson, M. and Hou, L., (1972) Biochem. Biophys. Res. Comm. 48: 749 - 756

Haber, J.E., (1975) In Spores VI (Gernhardt, P., Costilow, R.N. and Sadoff, M.L. Eds.) Am. Soc. Microbiol. Washington, 1974

Haber, J.E., Esposito, M.S., Magee, P.T. and Esposito, R.E. (1974) In Spores VI (Gernhardt, P., Costilow, R.N. and Sadoff H.L. Eds.) Am. Soc. Microbiol. Washington, 1974

Haber, J.E. and Halvorson, H.O. (1972) J. Bact. 109: 1022 - 1033

Harashima, S., and Oshima, Y., (1976) Genetics 84: 437 - 451

Hartig, A., and Breitenbach, M., (1980) Eurr. Genetics 1: 997 - 102

Hartwell, L.H., (1974) Bact. Revs. 38: 164 - 198

Hartwell, L.H., (1976) J. Mol. Biol. 104: 803 - 817

Hartwell, L.H., Culotti, J. and Reid, B. (1970) Proc. Nat. Acad. Sci. U.S.A. 66: 352 - 357

Hartwell, L.H., Culotti, J., Pringle, J.R. and Reid, B.J., (1974) Science 183: 46

Hartwell, L.H., Mortimer, R.K., Culotti, J. and Culotti, M., (1973) Genetics 74: 267 - 286

Hartwell, L.H. and Unger, M.W., (1977) J. Cell. Biol. 75: 422 - 435

Hashimoto, T., Gerhardt, P., Conti, S. and Naylor, H. (1960) J. Biophys. Biochem. Cytol. 7: 305 - 317

Herbert, D., Elsworth, R. & Telling, R.C. (1956) J. Gen. Microbiol. 14: 601 - 622

Hereford, L.M. and Hartwell, L.H., (1974) J. Mol. Biol. 84: 445 - 461

Herskowitz, I. and Oshima, S., (1981) In The Molecular Biology of the Yeast Saccharomyces (Strathern, N., Jones, E.W., Broach, J.R., Eds.) pp. 181 - 209, Cold Spring Harbor, New York, 1981

Hicks, J.B. and Herskowitz, I., (1976) Genetics, 83: 245 - 258

Hicks, J.B., Strathearn, J.N. and Herskowitz, I., (1977) In DNA Insertion Elements, Plasmids and Episomes (Burkhari, A., Shapiro, J. and Adhyer, S., Eds.) Cold Spring Harbor Press, New York, 1977

Hirschberg, J. and Simchen, G., (1977) Exptl. Cell Res. 105: 245 - 252

Hopper, A.K. and Hall, B.D. (1975) Genetics 80: 41 - 59

Hopper, A.K., Kirsch, J. and Hall, B.D., (1975) Genetics 80: 61 - 76

Hopper, A.K. and Mackay, V.L., (1980) Mol. Gen. Genet. 180:301 - 314

Hopper, A.K., Magee, P.T., Welch, S.K., Friedman, M. and Hall, B.D., (1974) J. Bact. 119: 619 - 628

Hunt, T., and Jackson, R.J., (1974) In Modern Trends in Human Leukaemia (Neth, R., Gallo, R.C., Spiegelmann, S. and Stohlman, F. Eds.) pp 300 - 307 J.F. Lehmann's, Munich

Hynes, M.J., (1974) J. Bact. 120: 1116 - 1123

Lorincz, A., and Carter, B.L.A., (1979) J. Gen. Microbiol. 113: 287 - 295

Mackay, V., and Manney, T.R., (1974a) Genetics 76: 255 - 271

Mackay, V., and Manney, T.R., (1974b) Genetics 76: 273 - 288

Magee, P.T., (1974) M of Biol. Reports 1: 275 - 281

Magee, P.T. and Hopper, A.K., (1974) J. Bact. 119: 952 - 960

Malone, R.E. and Esposito, R.E., (1981) Mol. Cell Biol. 1: 891 - 901

Malone, R.E., Golin, J.E. and Esposito, M.S., (1980) Curr. Genetics 1: 241 - 248

Mandelstam, J., (1969) Symp. Soc. Gen. Microbiol. 19: 377 - 402

Matile, P.H., Moor, H., and Robinow, F. (1969) in The Yeasts Vol I (Rose, A.H. and Harrison, J.H. Eds.) Academic Press, London 1969

Matur, A. and Berry, D.R. (1978) J. Gen. Microbiol. 109: 205 - 213

Mazon, M.J. (1978) J. Bact. 133: 780 - 785

Mazon, M.J. and Hemmings, B.A., (1979) J. Bact. 139: 686 - 689

McAlister, L. and Finkelstein, D.B. (1980) J. Bact. 143: 603 - 612

Miller, J.J., (1963a) Nature, London 198: 214 - 215

Moens, P.B., (1971) Can. J. Micro. 17: 507 - 510

Moens, P.B., Esposito, R.E. and Esposito, M.S. (1974) Exptl. Cell Res. 83: 166 - 174

Moens, P.B. and Rapport, E., (1971) J. Cell Biol. 50: 344 - 361

Monod, J., (1942) Recherches sur la croissance des cultures bacteriennes Paris: Hermann et Cie (Cited by Dawes & Thornley, 1970)

Mortimer, R.K. and Hawthorne, D.C. (1969) In The Yeasts Vol I (Rose, A.H. and Harrison, J.H. Eds.) pp 386 - 453. Academic Press, London, 1969.

Mortimer, R.K. and Hawthorne, D.C., (1975) In Methods in Cell Biology, XI (D Prescott ed.) pp 221 - 234. London, 1975.

Nagai, S., Yanagishima,N., and Nagai, H., (1961) Bact. Revs. 25: 404 - 426

Newlon, M.C., (1979) Mol. Gen. Genetics 176: 297 - 300

Newlon, M.C. and Hall, B.C., (1978) Mol. Gen. Genet. 165: 113 - 114

Ogur, M., Roshanmesh, A. and Ogur, S., (1965) Science 147: 1590

Ogur, M., St John, R. and Nagai, S. (1957) Science 125: 928 - 929

Opheim, D.J., (1979) J. Bact. 138: 1022 - 1025

Pelham, H.R.B. and Jackson, R.F., (1976) Eur. J. Biochem. 67: 247 - 256

Peterson, J.G.L., Kielland-Brandt, M.L. and Nilsson-Tillgren,J., (1979) Carlsberg Res. Comm. 44: 149 - 169

Piggot, P.J. and Coote, J.G., (1976) Bact. Revs. 40: 908 - 962

Pinon, R., (1977) Exptl. Cell Res. 105: 367 - 378

Pinon, R., (1978) Chromosoma 67: 263 - 274

Polakis, E.S., and Bartley, W., (1965) Biochem. J. 97: 284 - 297

Prakash, S., Prakash, L., Burke, W., and Montelone, B.A., (1980) Genetics, 94: 31 - 50

Pratje, E., Schulz, R., Schnierer, S. and Michaelis, G.,(1979) Mol. Gen. Genet. 174: 411 - 415

Pringle, J.R., (1978) J. Cell Physiol. 95: 393 - 406

Pringle, J.R. and Hartwell, L.H., (1981) In The Molecular Biology of the Yeast Saccharomyces (Strathern, N., Jones, E.W. and Broach, J.R. Eds.) pp 211 - 288 Cold Spring Harbor, New York 1981.

Rai, R., Piggott, J.R., and Carter, B.L.A. (1982) Abstract Soc. for Gen. Microbiol. 94th Ordinary Meeting, Cambridge, 1982 M7

Ramirez, C. and Miller, J.J., (1964) Can. J. Microbiol. 10: 623 - 631

Reed, S.I., (1980) Genetics 95: 561 - 577

Reed, S.I., Ferguson, J. and Groppe, J.C., (1982) Mol. Cell Biol. 2: 412 - 425

Rhaese, H.J., Scheckel, R., Groscurth, R. and Stamminger, G. (1979) Mol. Gen. Genet. 170: 57 - 65

Robinow, C.F. and Marak, J. (1966) J. Cell Biol. 29: 129 - 151

Roman, H. and Sands, S.M. (1953) Proc. Nat. Acad. Sci. USA 39: 171 - 179

Roth, R., (1973) Proc. Nat. Acad. Sci. USA 70: 3087 - 3091

Roth, R. and Halvorson, H.O. (1969) J. Bact. 98: 831 - 832

Roth, R., and Lusnak, K., (1970) Science 168: 493 - 494

Sherman, F., (1975) In Methods in Cell Biology, XI (Prescott, D. Ed.) pp 189 - 200. London, 1975.

Sherman, F. and Roman, H., (1963) Genetics 48: 255 - 261

Shilo, V., Simchen, G. and Shilo, B., (1978) Exptl. Cell Res. 112: 241 - 248

Shuster, J.R., (1982) J. Bact. 151: 1059 - 1061

Simchen, G., (1974) Genetics 76: 745 - 753

Simchen, G., Pinon, R. and Salts, Y., (1972) Exptl. Cell Res. 112: 241 - 248

Slater, M.L., Sharrow, S.O. and Gart, J.J., (1977) Proc. Nat. Acad. Sci. USA 74: 3850 - 3854

Snider, I.J. and Miller, J.J., (1966) Can. J. Biochem. 12: 485 - 488

Sogin, S.J., Chaffin, W.L. and Halvorson, H.O., (1975) In Spores VI (Gernhardt, P., Costilow, R.N. and Sadoff, H.L. Eds) Am. Soc. Microbiol. Washington, 1974.

Sprague Jr., G.F., Rine, J. and Herskowitz, I., (1981) Nature, 289: 250 - 252

Stark, H.L., Fugit, D. and Mowshowtiz, D.B., (1980) Genetics 94: 921 - 928

Strathern, J., Hicks, J. and Herskowitz, I., (1981)
J. Mol. Biol. 147: 357 - 372

Sudbery, P.E., Goodey, A.R. and Carter, B.L.A. (1980) Nature,
288: 401 - 404

Sumrada, R. and Cooper, T.G. (1978) J. Bact. 136: 234 - 246

Suomalainen, H., and Oura, E., (1971) In The Yeasts Vol II
(Rose, A.H. and Harrison, J.J. Eds.) pp 3 - 74
Academic Press, London 1971

Swartzendruber, D.E., (1977) Exptl. Cell Res. 109: 439 - 443

Taylor, J.M., (1979) Ann. Rev. Biochem. 48: 681 - 717

Thuriaux, P., Nurse, P. and Carter, B.L.A. (1978) Mol. Gen. Genet.
161: 215 - 220

Tingle, M.A., Kuenzi, M.T., Halvorson, H.O., (1974) J. Bact.
117: 89 - 93

Tingle, M.A., Singh-Klar, A.J., Henry, S.A. and Halvorson, H.O.,
(1973) Symp. Soc. Gen. Microbiol. 23: 209 - 243

Trew, B.J., Friesen, J.D. and Moens, P.B., (1979) J. Bact.
138: 60 - 69

Villa - Komaroff, L., McDowell, M., Baltimore, D. and Lodish, H.F.,
(1974) Meth. Enzymol. 30: 709 - 723

Vezinhet, F., Kinnaird, J.H. and Dawes, I.W. (1979) J. Gen.
Microbiol. 115: 391 - 402

Wejksnora, P.J. and Haber, J.E. (1978) J. Bact. 134: 246 - 260

Wheals, A.E., (1982) Mol. Cell Biol. 2: 361 - 368

Wright, J.F., Ajam, N., and Dawes, I.W. (1981) Mol. Cell. Biol. 1: 910 - 918

Wright, J.F. and Dawes, I.W., (1979) FEBS Letts. 104: 183 - 186

Ycas, M., Sugita, M. and Bensam, A., (1965) J. Theor. Biol. 9: 444 - 470

Zacharski, C.A. and Cooper, T.G., (1978) J. Bact. 135: 490 - 497

Zubenko, G.S. and Jones, E.W., (1981) Genetics 97: 45 - 64

## ADDITIONAL REFERENCES

Calvert ,G.R. and Dawes,I.W. (1981) Abstract, 92nd ordinary meeting of the British Society for General Microbiology, Edinburgh, 1981.

Calvert,G.R. and Dawes,I.W. (1984) In press, manuscript accepted by Journal for General Microbiology.

Dawes,I.W. and Calvert,G.R. (1984) In press, manuscript accepted by Journal for General Microbiology.

Miller,J.J. (1963b) Can. J. Microbiol. 9 259.

Weir-Thompson,E.M.,Calvert,G.R.,and Dawes,I.W.(1982) Absract, 12th International Symposium of Yeast Genetics and Molecular Biology, Montpellier,France,1982

H26 DEVELOPMENTALLY REGULATED CHANGES IN TRANSLATABLE mRNA DURING YEAST SPORULATION, E. M. Weir-Thompson, G. R. Calvert and I. W. Dawes, Department of Microbiology, University of Edinburgh, Edinburgh, EH9 3JG, U.K.

The biochemical events that underlie the processes of meiosis and spore formation in yeast are, as yet, poorly understood, but there is evidence that there are some changes in cellular proteins during these processes and that a number of these changes are or may be sporulation specific (1, 2). These could have been due to modification of existing proteins or to new gene expression.

We have shown that there is a concomittant change in the availabilty of particular mRNA species by analysing the translatable mRNA population of sporulating a/α and isogenic non-sporulating a/a, α/α strains under sporulation conditions. RNA was prepared from cells at different times after resuspension in sporulation medium and was used to programme an optimised rabbit reticulocyte cell-free system. The resultant *in vitro* translation products have been analysed by one- and two-dimensional gel electrophoresis and autofluorography of the [$^{35}$S]-methionine labelled polypeptides. So far, we have observed a number of changes in the mRNA content of the yeast cells during sporulation, some of which appear to be specific to sporulation. The changes in translatable mRNA species, their specificity to sporulation and their timing will be outlined.

1. Wright, J. F. & Dawes, I. W. (1979). FEBS Letts. *104*, 183-186.
2. Wright, J. F., Ajam, N. & Dawes, I. W. (1981). Molec. Cellular Biol. *1*, 910-918.

H24 GENETIC INVOLVEMENT IN SPORULATION INITIATION, Ian W. Dawes and Geoffrey R. Calvert, Department of Microbiology, University of Edinburgh, Edinburgh, EH9 3JG, U.K.

To date, several different classes of mutation that affect the initiation of sporulation have been described (1, 2). These mutations also affect the cell division cycle and, in some cases, the ability of the cells to survive starvation is also impaired. The mutations include those leading to conditional derepression of sporulation (*spd1, spd3, spd4, cdc25, cdc35*) or to partial or complete asporogeny (*spo50, spo51, spo53, cdc28?, tra3*).

Complementation and linkage analysis have shown that *spd1, spd3, spd4, spo50, spo57, spo53, cdc25, cdc28* and *cdc35* are all unlinked genes belonging to separate linkage groups. The *spd* mutations are all unstable, giving rise to revertants at a frequency of $10^{-3}$ to $10^{-4}$ per cell, and in most cases the reversion is due to the acquisition of an *spo* mutation that is epistatic to the *spd*. Further tests of epistasis of all these mutations in suitable constructed homozygous diploids has been used to construct a model for the participation and interaction of all the above genes in the processes of cell division, sporulation and survival of starvation.

1. Shilo, V., Simchen, G. & Shilo, B. (1978). Exp. Cell Res. *112*, 241-248.
2. Dawes, I. W. (1975). Nature *255*, 707-708.

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92nd Meeting of British Society for General Microbiology, Edinburgh, Sept. 1981.

## New Gene Expression During Sporulation in Saccharomyces cerevisiae.

G.R. Calvert and I.W. Dawes.

Dept. Microbiol., Univ. Edin., Edinburgh EH9 3JG.

The appearance of new polypeptide species during sporulation in S. cerevisiae has been shown by two-dimensional polyacrylamide gel-electrophoresis. The method used to show these changes would have detected mofifications to pre-existing polypeptides as well as changes arising from de novo synthesis. Pulse labelling studies have been unable to detect de novo synthesis at the level of 400 or so polypeptides resolvable by two-dimensional gel electrophoresis. However these studies may not have taken into account the lack of homogeneity in the abilities of cells at different stages of sporulation in the culture to take up labelled amino acids. To detect de novo synthesis of polypeptides, total RNA samples extracted from yeast cells at various stages of sporulation were incubated in a cell free protein synthesisisng system derived from rabbit reticulocytes. Even by one dimensional gel-electrophoresis, several sporulation-specific protein products were detected, indicating the appearance of several new species of RNA during sporulation.

Cloned DNA fragments containing sporulation specific sequences of yeast DNA have been used as probes for their complementary messenger RNAs using a hybridisation-inhibition method in the cell free protein synthesising system. Messenger RNAs that hybridise to complementary DNA are unable to direct protein synthesis in vitro.

## Mutants of Saccharomyces cerevisiae Defective in the Initiation of Sporulation.

I.W. Dawes and G.R. Calvert.

Dept. Microbiol., Univ. Edin., Edinburgh EH9 3JG.

Diploid strains of S. cerevisiae homozygous for the spd (sporulation derepressed) series of mutations initiate sporulation at high rates when grown on non-fermentable carbon sources. Genetic analysis indicates that mutations at several loci confer this phenotype.

A high rate of reversion to growth on non-fermentable carbon sources in strains carrying the spd 1 mutation has been shown to involve an unlinked suppressor mutation at one of two loci designated spo 50 and spo 51. The phenomenon does not appear to involve nonsense suppressors. Diploid strains homozygous for these mutations are unable to initiate sporulation under any conditions tested. The mutations spo 50 and spo 51 are epistatic to spd 1 in homozygous diploids.

These mutations confer an altered phenotype on strains carrying them. To a certain extent during vegetative growth and to a pronounced degree when starved, these strains assume an aberrant phenotype with clumps of cells having long filamentous extrusions and large irregularily shaped bodies with apparently continuous cytoplasm. The strains also show marked loss of viability when starved. The production of these aberrant irregulary shaped cells is also found in strains carrying the cdc 28 cell-cycle initiation mutation of Hartwell, and the genetic relationship of this mutation to ours is being tested.